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Edited at The University of Texas Medical Branch, Galveston

THE TRANSIENT VIABILITY OF THE SPOTTED FEVER RICKETTSIA IN TRIATOMA GERSTAECKERI*

LUDWIK ANIGSTEIN AND ARDZROONY PACKCHANIAN

Although the tick-borne nature of the spotted fever group of diseases has been fully established, the possibility of transmission of this disease under certain circumstances by incidental vectors cannot be completely eliminated. Many species of blood-sucking reduviid bugs, chiefly *Triatoma,* are widely distributed in southwest United States and in Central and South America. These insects feed on various species of animals—wood rats, opossums, armadillos, cattle, horses, dogs, cats, rabbits, etc. (Packchanian, 1939, 1940). Inasmuch as these animals also serve as hosts to the tick, *Amblyomma americanum*, the most prevalent vector of spotted fever in Texas, the question arises whether the co-existence of spotted fever and species of *Triatoma* in Texas will represent a potential epidemiologic problem.

The possible role of Triatominae in the transmission of rickettsiae was first investigated by Dias and Martins (1937), who found that the etiologic agent of Brazilian spotted fever did not persist in the gut of *Panstrongylus megistus* longer than 48 hours.

Philip and Dias (1938) demonstrated that the longest survival period of *Dermacentroxenus rickettsii* was about 24 to 72 hours in the following insects: *Eutriatoma uhleri, Triatoma protracta, T. infestans, Panstrongylus megistus,* and *Rhodnius prolixus.*

The survival of murine typhus rickettsiae has been observed for 16 days in *T. infestans* (Violle and Sautet, 1938) and for at least 33 days in *T. barberi* (Varela and Mazzotti, 1943). Nauck and Zumpt (1940) were unable to transmit epidemic typhus through the bite of *T. rubrofasciata,* although this insect may harbor living rickettsiae for some time. Veintemillas (1941) claims to have obtained typhus transmission by *T. infestans.*

*From the Rickettsial Research Laboratory and Department of Bacteriology and Parasitology, The University of Texas Medical Branch, Galveston, Texas. This study was supported by funds supplied by USAF School of Aviation Medicine, Contract No. AF(607)-43. The writers express their thanks to Miss Betty Celaya and Miss Dorothy Whitney for their interest and aid in this study. Received for publication July 20, 1952.

Observation of natural infection with a rickettsia-like organism in a reduviid bug was reported by Webb (1940). These rickettsiae, seen in smears from the tissues of *T. rubrofasciata* intracellularly and intranuclearly, were maintained in guinea pigs and produced pathological lesions in laboratory animals (Steinhaus, 1946). Although the intranuclear findings strongly suggest *Dermacentroxenus*, the true identification of the organisms was not proved.

Packchanian (1948) demonstrated that several species of pathogenic and non-pathogenic trypanosomes, excluding *Trypanosoma cruzi*, failed to multiply or survive more than 10 days in the gut of *Triatoma gerstaeckeri*.

In view of the co-existence of the reduviid bugs and spotted fever in Texas, as well as of common mammalian hosts for the *Triatoma* and ticks, an experimental study on the survival of spotted fever rickettsia in *Triatoma gerstaeckeri* was undertaken.

Material and Methods

Infective agent: The Sheila-Smith strain of Rocky Mountain spotted fever was used as the infective agent. When guinea pigs are inoculated intra-abdominally with this strain, a clear-cut febrile reaction, typical scrotal lesions, and a fatality rate of about 75 per cent are invariably produced. Spleens of infected guinea pigs were kept in dry ice and used for inoculations.

Species of Triatoma: One hundred laboratory reared, medium sized nymphs and adult male and female *Triatoma gerstaeckeri* were used for possible transmission of Rocky Mountain spotted fever. The parents of these insects were collected in Texas (Packchanian, 1939).

Method of infecting Triatoma: Several groups of fasting *T. gerstaeckeri* in suitable containers were allowed to feed at intervals on experimentally infected guinea pigs, usually on the third day of high fever. These fasting insects readily fed on the guinea pigs through gauze, and within about an hour, all were engorged with the infective blood. Each insect usually obtained about 0.3 to 0.5 ml. of blood.

As a control, while the insects were feeding, about 0.3 ml. of blood was removed from each infected guinea pig, and inoculated intra-abdominally into normal guinea pigs to ascertain

the subsequent rickettsiemia in the feeder. This amount of infective material corresponds to that withdrawn by the fully engorged insect.

All the infected insects were kept at about 25° Centigrade.

Examination of infected Triatoma for virulence and viability of spotted fever rickettsia: The survival time of the rickettsia in *T. gerstaeckeri* was determined by injection of tissues and blood suspension of the insects into guinea pigs at intervals from 10 minutes to 34 days, following the infective blood-meal, and in two instances, 233 days and 255 days. Two to nine *Triatoma* from each group were used for each guinea pig inoculation. These insects were placed in a large glass funnel and repeatedly rinsed with sterile saline. They were then crushed and triturated in a sterile mortar with one milliliter of saline for each *Triatoma.* About two milliliters of the suspension was inoculated intra-abdominally into each experimental animal.

These inoculated animals were observed daily up to three weeks for the symptoms of spotted fever and later for immunity against reinfection with this disease.

Experimental Data

A. *The positive findings:* All the experimentally infected *Triatoma* produced typical Rocky Mountain spotted fever when inoculated into susceptible test animals within 10 minutes to nine hours following the infective meal.

The first series of this study consisted of three medium sized nymphs which were engorged with blood from an infected guinea pig. Five minutes after the feeding, these insects were crushed, pooled, and the suspension was inoculated into two normal guinea pigs. Both animals responded with high fever after three to five days' incubation period and manifested typical spotted fever symptoms. These guinea pigs developed immunity against reinoculation with *D. rickettsii* and were discarded 15 days later.

In the next experiment, two nymphs and two adult insects were used. These were crushed about ten minutes following the infective blood-meal and inoculated into a normal guinea pig. This animal developed spotted fever, recovered, and developed complete immunity to reinoculation.

The 30-minute survival test was performed with four nymphs. These were crushed in saline and the suspension was inoculated into a normal guinea pig. This animal contracted spotted fever and succumbed to the disease in nine days.

Another series of experiments consisted of four nymphs. These were inoculated into a guinea pig four hours after the infective blood-meal. The inoculated animal contracted spotted fever and died in nine days.

A second four-hour interval test was conducted with four nymphs and two adult *Triatoma*, which were also inoculated into a guinea pig. This animal manifested typical clinicial symptoms of spotted fever but recovered, and developed immunity to reinoculation.

The next positive findings consisted of two nymphs and three adults. These were inoculated nine hours after the infective blood-meal into a normal guinea pig, producing an acute fever after an incubation period of six days. The fever lasted five days and the disease terminated fatally 11 days following the initial inoculation.

B. *The negative findings:* The following series of experiments which were conducted on the infected *Triatoma* one to 255 days after the infective meal all proved to be negative. None of the inoculated test animals contracted the disease or developed immunity.

One nymph and three adult *Triatoma* were crushed 24 hours after the infective meal, and the suspension was inoculated into two normal guinea pigs. Both of these animals failed to show any clinical symptoms of spotted fever. When these guinea pigs were reinoculated three weeks later with spotted fever, the animals showed no immunity. In other words, no viable rickettsiae remained in the *Triatoma* 24 hours after their blood-meal. Similar negative results were obtained after a 48-hour interval with two engorged adult *Triatoma*. No elevation of the animals' temperature was noted in this latter series during two weeks' observation. As in previous cases, the challenge with spotted fever showed no immunity.

Finally, longer intervals of four, six, eight, and 34 days' duration for survival tests were chosen and all proved negative. In two instances, a much longer period (233 to 255 days) for reinfection studies was carried out. One hundred and seven days

after the first infective blood-meal, three nymph *Triatoma* were re-infected. During the interval between infective meals, the insects were allowed to feed on normal rats or mice. Two hundred and thirty-three days after the original infective feeding, these nymphs were crushed and inoculated into a normal guinea pig. This animal failed to produce clinical evidence of spotted fever or immunity. Similarly, a group of five nymphs, with a time lapse of 255 days, was inoculated into a guinea pig, yielding negative results.

A total of 39 nymphs and five adult infected *Triatoma* were used in this latter series. Prior to inoculation, some of these insects were fed on normal mice and a rat several days after their infective meals. This was done because Spencer and Parker (1923) suggested that "dormant" rickettsiae can be reactivated by such a procedure.

Discussion

The attempt to include *Triatoma* into the natural cycle of spotted fever where the tick is the primary arthropod and from which the disease originates in nature, may be interpreted as an experimental deviation from the natural channels.

Baker (1943), in his analysis of "The Typical Epidemic Series," states that diseases which may be thought of as originating in arthropods seem to specialize in a series of steps, eventually reaching their peak in the typical epidemic (Steinhaus, 1946). *Triatoma*, as a close associate of animals including man, enters the present picture as a secondary arthropod. The latter could under special circumstances accomplish a reversible transmission of such organisms as rickettsiae between an animal reservoir and man. Should this be the case, the *Triatoma* as a secondary arthropod would only be incidental, contrary to its fundamental role as a primary arthropod in Chagas' disease. We may only speculate as to whether the spontaneous infection with a rickettsia, possibly of the spotted fever type of *T. rubrofasciata*, as observed by Webb (1940), actually offers an example of such deviation from the natural cycle. In the light of previous observations on the relatively long survival of murine typhus rickettsiae in *T. infestans* (Violle and Sautet, 1938), and *T. barberi*, at least 33 days (Varela and Mazzotti, 1943), the feasibility of typhus transmission would exist contrary to the

limited survival time of spotted fever rickettsiae in *T. gerstaeckeri* which renders transmission questionable.

The present study demonstrates that *Dermocentroxenus rickettsii* survive an even shorter period of time in *Triatoma.* The exact survival time of *Dermocentroxenus* in *Triatoma gerstaeckeri* has not been determined, but it is apparently longer than nine and shorter than 24 hours under the described experimental conditions.

It is noteworthy that the intestine of *Triatoma,* except for the symbiotes (Steinhaus, 1946), is almost invariably free from bacteria. Although *Trypanosoma cruzi* live and multiply luxuriantly in the gut of various species of *Triatoma,* it is significant that closely related trypanosomes such as *Tr. lewisi, Tr. duttoni,* and pathogenic trypanosomes such as *Tr. brucei, Tr. gambiense, Tr. hippicum,* and *Tr. rhodesiense* will not multiply nor survive more than 10 days in the gut of *Triatoma* (Packchanian, 1948).

It is, however, interesting to note that Duncan (1926), who first demonstrated that an antibacterial principle exists in the guts of insects and arachnids, believed that this active principle is formed in the stomach. However, he was unable to prove whether this principle is formed as a result of secretion of the gastric cells or as a result of the process of digestion.

Similar findings were reported by Anigstein, Whitney, and Micks (1950), who suggest the possible bactericidal role of metabolic processes, especially of substances derived from digested blood.

Summary

Dermocentroxenus rickettsii remained virulent in *Triatoma gerstaeckeri* at least nine hours. Guinea pigs inoculated within this period following the infective blood-meal showed typical symptoms of spotted fever, died, or developed immunity against the disease.

Guinea pigs inoculated with *Triatoma* one, two, four, six, eight, nine, 34, 233, and 255 days after the infective blood-meal, failed to develop any symptoms or immunity against *D. rickettsii.* All the animals survived.

It is concluded that *T. gerstaeckeri* do not play any primary role in the transmission of spotted fever.

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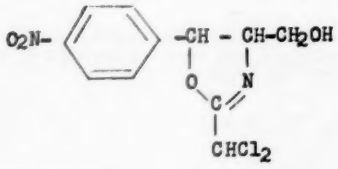
ANTIRICKETTSIAL ACTIVITY OF A SYNTHETIC COMPOUND RELATED TO CHLORAMPHENICOL*

LUDWIK ANIGSTEIN AND DOROTHY M. WHITNEY

The synthetic approach to chemistry of antibiotics has yielded so far only one product, namely, the aromatic nitro compound, chloramphenicol (Controulis *et al.*, 1949). This purely chemical substance which displays an antimicrobial specificity of the same order as the fermentation product does not strictly comply with the definition of antibiotics restricted to products of living organisms. It can be anticipated that the synthesis of other chemically well-defined agents identical with biological products will justify a revision of our concept of antibiotics. One of these compounds has been recently synthetized by Hoffmann-La Roche, Inc., and designated by them temporarily as Ro 1–6600 (personal communication).

The new compound is a colorless, crystalline, bitter tasting powder, slightly soluble in water at 20° C., more readily soluble in hot water, alcohol and propylene glycol. The chemistry reveals a molecular structure related to that of chloramphenicol. Both Ro 1–6600 and chloramphenicol are characterized by p-nitrophenyl in the aromatic portion. In Ro 1–6600, chlorine is attached to the oxazole ring whereas in chloramphenicol, oxazole is replaced by an aliphatic chain with chlorine in the dichloroacetamide. In essence, Ro 1–6600 is an internal anhydride of chloramphenicol.



Ro 1–6600: D-2-Dichloromethyl-5-(p-nitrophenyl)-4-oxazoline-methanol. The maximal tolerated peroral dose (MTD) for mice

*From Rickettsial Research Laboratory, The University of Texas Medical Branch, Galveston. Presented at the meeting of Texas Branch of Society of American Bacteriologists, May 16, 1952, Galveston, Texas. The compound investigated in this study has been kindly supplied by Hoffmann-La Roche, Inc.

is 1,000 mg./Kg., the LD 50, 1,375 mg./Kg. with 3,000 mg./Kg. and 5,000 mg./Kg. respectively for rats. According to Hoffmann-La Roche, the red blood cell counts were normal for rats fed 150 mg./Kg. for 2 weeks and slightly diminished in those fed 450 mg./Kg. The white blood cell counts were normal. No toxicity data were available for the guinea pig which, as the animal of choice for experimental spotted fever, has been used in the prsent study. Our own observations point to a peroral MTD of about 1,000 mg./Kg. per day over a period of 8 days. Data supplied by Hoffmann-La Roche indicate that Ro 1–6600 is an efficient curative agent in infections due to *S. typhosa*, *S. schottmuelleri* and *E. coli* in mice in single oral doses. The results were comparable to those obtained with chloramphenicol. No clinical trials in human cases were reported as yet.

Material and methods. Only peroral administration of the drug mixed with Purina rabbit pellets was used in the treatment of guinea pigs (450 to 600 g. each). This procedure is, however, handicapped by the animal's considerable aversion to the diet containing Ro 1–6600, particularly when its content reaches therapeutic levels. After various trials, pretreatment of the animals prior to infection resulted in a satisfactory intake. With the content in diet from 0.5% to 2.5%, the drug intake varied from 100 to 500 mg. per guinea pig in 24 hours. Pretreatment of guinea pigs usually began 2 or 3 days before infection with spotted fever, the drug intake continued during the incubation period of the controls and then was subject to variations depending on the individual fever reactions. In addition to the voluntary intake in diet, forced-feeding with a suspension of 100 mg. Ro 1–6600 in milk was occasionally used. The well established Sheila-Smith strain of Rocky Mountain spotted fever showing a clear-cut high and continuous fever after 3–4 days incubation and a fatality rate of about 75% was used as the infective agent. Spleens of infected animals were maintained in dry ice and used for intra-abdominal inoculations.

Experiments and results. In most instances inoculation with spotted fever followed a pretreatment with Ro 1–6600 in diet for 2–3 days in order to condition the animals to the drug and also to establish whether chemoprophylaxis would contribute to a suppression of clinical symptoms, as shown with chloramphenicol (Smadel *et al.*, 1949). While the drug was continued, a

definite suppression of clinical symptoms as evidenced by a normal temperature curve was observed in test animals. When, however, the drug was removed from the diet, febrile responses usually followed (Fig. I). These reactions which flared up some-

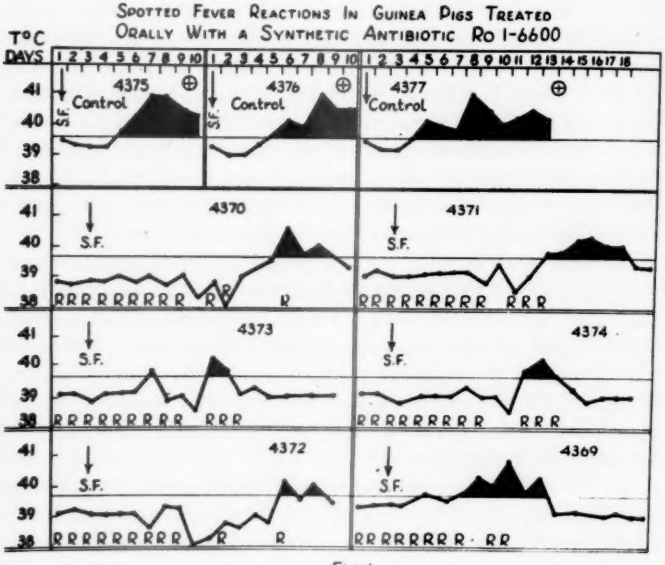


Fig. I

time after a quiescent period of 13 days were indicative of a rickettsiostatic action of the drug similar to chloramphenicol and aureomycin (Smadel *et al.*, 1949; Anigstein *et al.*, 1948). In contrast to the untreated controls, these febrile attacks were of mild or abortive character, in many cases lasting only one day. After treatment was resumed, fever could be brought down and the temperature remained normal. This was particularly noticeable at later stages of the disease when the rickettsiostatic effect of the antibiotic was enhanced by the immune mechanism. On the other hand, when the treatment was interrupted at an early stage of the suppressed infection a moderate fever developed. In these cases, similar to chloramphenicol and scrub typhus (Sma-

del *et al.*, 1949), the immune response was apparently not adequate for a final suppression of symptoms. A reinoculation of the test animals with virulent spotted fever material 3 weeks after the primary infection proved their complete immunity regardless of the previous fever reactions even of one day duration. In another series of experiments in which the test guinea pigs developed fever simultaneously with the controls, additional peroral administration of 200 mg. Ro 1–6600 in 2 divided doses per day was instituted (Fig. II). In interrupting and resuming

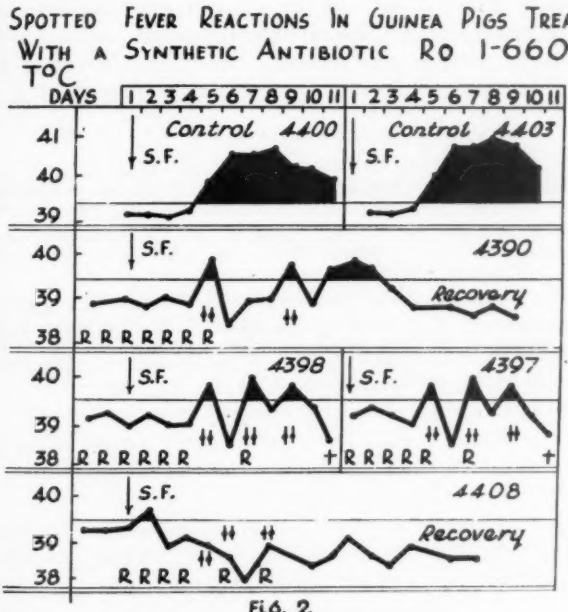


Fig. 2

R—Antibiotic in food 2.5%
↓—100 mg orally by tube

the treatment at daily intervals an intermittent type of fever was produced; however, due to poor food consumption a considerable loss in weight followed and some animals died from cachexia. Other guinea pigs of the same series recovered including a case of complete suppression of clinical symptoms and fever.

Summary and conclusions. A new synthetic antibiotic chemically related to chloramphenicol produced by Hoffmann-La Roche was tried in experimental spotted fever in guinea pigs. The drug was incorporated in diet up to 2.5%, the daily intake varying from 100 to 500 mg./day/guinea pig. Treatment of guinea pigs with the drug preceding and following the infection with spotted fever showed a clinical suppression of the disease. This was evidenced either by a complete absence of fever or by abortive attacks of fever of short duration. The recovered guinea pigs when challenged with spotted fever showed complete immunity to reinoculation. The preliminary results indicate rickettsiostatic activity of the synthetic antibiotic; however, the effective time-dose relationship has not yet been established.

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WOUND HEALING PROPERTIES OF THE BLOOD HYDROLYSATE, SANGUININ

MADERO N. BADER, D.V.M.*

The application of blood or its components in the treatment of wounds has been the subject of several investigations. Observations reported in this country (Naide, 1943; Moorhead and Unger, 1943) as well as by European investigators (Richter, 1941; Kreyer, 1942; Allgöwer, 1949) point to the beneficial effect of direct application of red blood cells on chronic ulcerative wounds or suppurative burns. Red cells discarded from the preparation of human blood plasma were used as a concentrate of semiliquid consistency in surgical dressings by Moorhead and Unger (1943). Burns, infected and non-infected wounds, and certain ulcerations have been benefited in the experience of these authors.

In another series, fresh human blood was applied by dropping or spraying on leg ulcers in patients with peripheral vascular diseases (Naide, 1943). Although these ulcers are difficult to cure, and sometimes incurable, by this method ischemic ulcers were healed after epithelization. Furthermore, dried red blood cells were recommended by Seldon and Young (1943) who realized the difficulties in keeping the semiliquid material in contact with the wound. It is to be noted that the interest to utilize human blood for wound healing developed during World War II as a problem of military medicine. Although the blood material for wound treatment was prepared in various forms, such as red cell paste (Orbach, 1946), dry red cells or blood cell jel (Kanski, 1946; Allgöwer, 1949), the red cells were chemically intact.

In the present work, an antibacterial product obtained from red blood cells of cattle, first reported and prepared by Anigstein, Whitney and Micks (1950), and further investigated by Whitney, Anigstein and Micks (1950) was furnished by these investigators to the author for treatment of surgical wounds of

*From the Keigan-Bader Small Animal Clinic, 2507 Bissonnet, Houston, Texas. Received for publication July 2, 1952.

dogs. Recent studies showed a suppressive effect of Sanguinin on streptococcal infection in mice (Micks, Whitney and Anigstein, 1951). In addition to the antibacterial properties of Sanguinin, a marked lowering of the liver catalase production in mice was lately revealed by Cline (1951).

Material and methods. Sanguinin is essentially a product of red blood cells submitted to enzymatic proteolysis by trypsin (1, 7, 12). After dialysis the liquid product is lyophilized to a dark brown amorphous powder. The trial of Sanguinin in dog surgery was made on 10 dogs of various breeds, whose ears were clipped in the hospital. The product was applied locally and directly on the cut surface of one ear of each dog by sprinkling the powder daily or every second day. Since the wound is open to contamination, pus formation and a purulent viscous exudate from the cut surface ordinarily follows. The time required for healing of clipped ears ranges usually from 2 to 3 weeks. This was the course of the process in the untreated ear of each dog which served as a control to the Sanguinin treated one.

Results. Except for 2 Boxer dogs in which no difference in healing time as compared with the control ears was noted, a striking acceleration of wound healing was observed in the remaining 8 dogs. The results were particularly spectacular in dog No. 7, where the treated cut surface healed completely in 6 days after application of Sanguinin, as compared with 16 days period of the untreated wound (Table 1). Following the first application of Sanguinin a dark crust is formed, sealing off the wound. Before each following application the crust was removed, exposing the fresh, moist and bright red granulation tissue. In the majority of cases a clear viscous exudate covered the tissue although in some a serous purulent exudate was discharged. A general and characteristic appearance was the abundant formation of granulation tissue as compared with the controls in which granulation tissue was poor during the period of observation. To the contrary, granulation tissue was stimulated in the treated cases with the exception of one. In some wounds granulation tissue was abundant to the extent that it interfered with epithelization. For this reason and due to the excessive formation of granulation tissue in some cases, treatment with Sanguinin had to be interrupted to prevent possible contractures of the ears.

TABLE 1

Topical Use of Sanguinin in Dog Surgery (Clipping of ears)

No.	Breed	Age	Sex	Operation Date	First Application	Number Applications	Healing Time in Days		Gen. Appearance of the Wound		Granulation Tissue	
							Treated	Control	Treated	Control	Treated	Control
1	Boxer	11 weeks	M	Jan. 2 1951	Jan. 3 1951	4	30	30	Fair	Fair	None	None
2	Boxer	3½ months	F	Dec. 26 1950	Dec. 28 1950	5	10	14	Good	Fair	Slight	None
3	Boxer	4½ months	M	Dec. 16 1950	Dec. 18 1950	6	11	18	Good	Fair	Moder.	None
4	Dober-man	3 months	M	Jan. 2 1951	Jan. 3 1951	6	11	15	Good	Fair	Slight	None
5	Boxer	3 months	M	Jan. 2 1951	Jan. 3 1951	3	18	18	Ex-cellent	Fair	Exces-sive	None
6	Boxer	9 weeks	M	Oct. 21 1950	Oct. 22 1950	5	12	20	Ex-cellent	Fair	Moder.	None
7	Boxer	11 weeks	M	Nov. 17 1950	Nov. 18 1950	6	6	16	Ex-cellent	Fair	Exces-sive	Slight to None
8	Dober-man	3 months	M	Oct. 20 1950	Oct. 22 1950	7	10	19	Ex-cellent	Fair	Slight	None
9	Boxer	11 weeks	M	Jan. 14 1951	Jan. 15 1951	3	17	19	Good	Fair	Moder. Excess.	None
10	Boxer	12 weeks	M	Jan. 14 1951	Jan. 15 1951	3	18	21	Good	Fair	Slight	None

REMARKS: Sanguinin was applied locally and directly on the cut surface of the ear by sprinkling the powder every day or every second day. The stimulation of the granulation tissue and rapidity of healing were striking except in 2 cases where no difference in healing as compared with the controls was found. No toxic or any untoward local or systemic effects of Sanguinin were noted.

In addition to the presented surgical wounds, the following case is reported by S. R. Kay, D.V.M., Galveston, Texas:

"Hound, male, 4 years, 50 pounds, large abscess, inguinal region, probably an acute exercerbation of a chronic process. Size of abscess, 4 inches diameter (medium size orange). Opened by routine method for drainage. No flushing of the cavity. About 250 ml pus evacuated. Prior treatment, manual examination revealed presence of many small pockets with fibrinous (reticular material). Treatment: Gauze pack soaked in Sanguinin solution (0.1 gm in 10 ml saline). Pack removed after 18 hours, lighter in color, foul odor. Complete absence of wound discharge. No further treatment until 24 hours later when wound was swabbed with Sanguinin. Granulation tissue had progressed so rapidly that patient was discharged. Usual pus discharge takes 4–6 days."

Dr. Kay's observations have been duplicated by the author in the treatment of abscesses with Sanguinin in cats. One cat had an acute abscess on the back over the sacral region; the other cat had an abscess on the shoulder. Both suppurating processes were apparently due to cat fights. The abscesses were of large size, about 3 inches in diameter and filled with greenish gray, thick pus. After incision and drainage the cavities were treated with a single dose of 0.1 gm. Sanguinin powder. Approximately 2 cc. of saline were used to rinse out the vial and this was poured into the wound. The next day the wounds showed a clean, healthy appearance with complete absence of pus. This was followed by an uneventful recovery and healing in 5 days.

Discussion and summary. The effect of direct application of red blood cells to wounds has been interpreted in various ways. The nutritional factor supplied by blood paste to the tissue deficient in adequate circulation was stressed by Murray and Shaar (1944). Orbach (1946) believes that a more important factor seems to be the protective action of the blood after formation of a blood crust, under which epithelization takes place.

Although the clinical material under our observation is limited, evidence is clear than Sanguinin in lyophilized form as applied directly to surface wounds or to abscess cavities, shortened considerably the healing process. The mechanism of this action is still obscure inasmuch as the chemical nature of Sanguinin requires elucidation. However, the previously demonstrated antibacterial property of Sanguinin can be credited with the rapid cleaning of the wounds. Furthermore, we can visualize that the

luxuriant growth of granulation tissue and rapid epithelization are apparently due to growth stimulating factor or factors inherent to Sanguinin and liberated by hydrolysis of the erythrocytes. The recent observation by Cline (1951) that Sanguinin displays anti-enzymatic properties in lowering the production of liver catalase will perhaps prove of value in the further analysis of the intrinsic characteristics of Sanguinin.

Conclusions. Sanguinin, a blood product obtained by enzymatic hydrolysis of red blood cells, when applied topically in open, contaminated and purulent wounds of the skin and in subcutaneous abscesses, showed debridement and healing effect. This includes purification of contaminated cut surfaces, stimulation of granulation tissue and shortening of the healing time.

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pH VARIATION DURING CULTURE DEVELOPMENT OF *TETRAHYMENA GELEII*

IBEN BROWNING AND MADELEINE S. BRITTAIN*

In our program of reproduction studies on cultures of the ciliated protozoan *Tetrahymena geleii*, we have found a progressive change of pH which must be taken into account. Wingo and Anderson (1951) have demonstrated a variation in maximum reproduction rate in this particular strain of Tetrahymena with change of initial pH. Their paper may be referred to for an analysis of previous work on the subject.

It was our purpose to measure the pH of cultures during development, and to correlate it with other observable phenomena.

Methods

The organisms, media, procedures and tabulations of data were the same in this experiment as in the series described by Browning, Brittain and Bergendahl in this journal. The additional procedure was to measure the pH of each aliquot sample with a Beckman pH meter.

Data and Results

In view of the fact that the pH has been found to vary during the progress of an experiment, the first obvious way to show results is to graph pH against time (see Figure I). Such a graph shows very little, but may be taken to show that time is not the element which produces a change in pH.

A second presentation is to graph pH against number of organisms in a typical experiment (see Figure II). It is obvious at this point that the end of the reproductive (increasing) phase of culture development is characterized by a change in effect on pH. Since our efforts are to analyze reproduction (not death), we confined our subsequent efforts to the reproductive phase. In graphing pH and population data from a series of experiments

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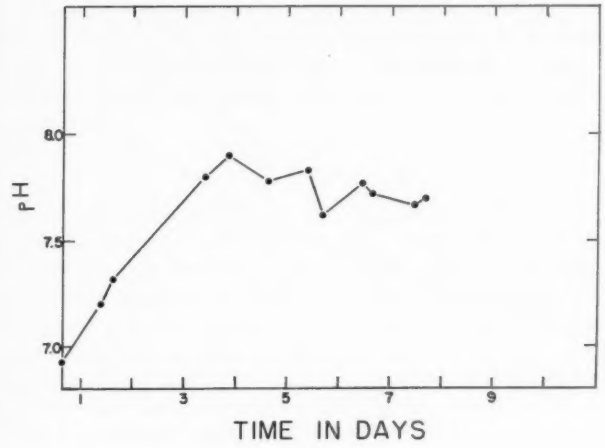

FIGURE I

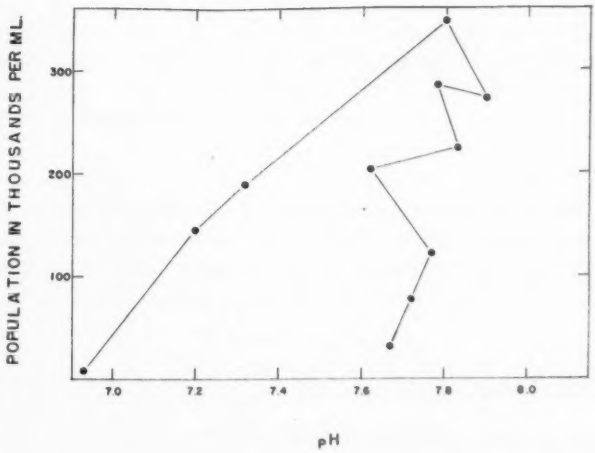

FIGURE II

which differed with respect to oxygen concentration in the bubbled gas, it became obvious that these parameters are related (see Figure III).

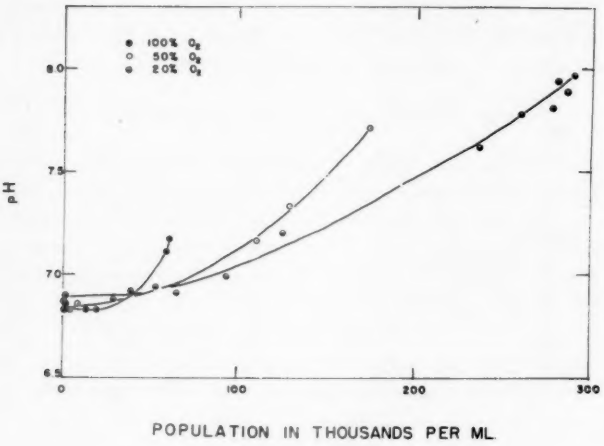

FIGURE III

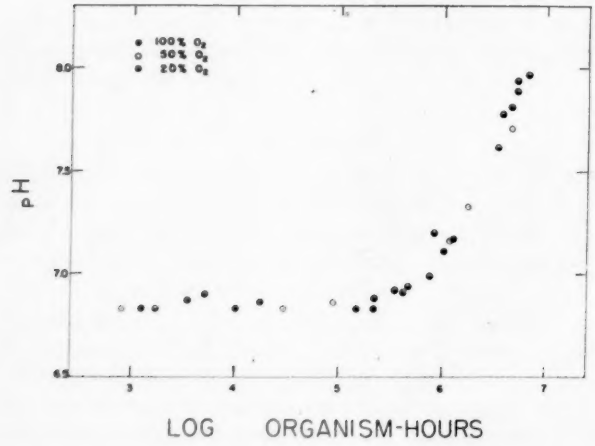

FIGURE IV

The next comparison was made between change of pH and the total number of *organism-hours* spent in the medium. To accomplish the comparison, the area beneath each graph of popu-

lation against time was integrated with a polar planimeter. The points in Figure IV show such a compilation of data. Even though these data were taken from cultures grown under various compositions of bubbled gas, the plot shows a coherent relationship.

Discussion

These data show that there is a continuous pH shift during the development of a Tetrahymena culture under our conditions. Wingo and Anderson (1951) have demonstrated that the rate of reproduction is dependent upon initial pH. It may logically be inferred, therefore, that a developing culture modifies its own reproduction rate by altering its hydrogen ion concentration. Such a self-induced modification of reproduction rate must be additive with other factors which even more profoundly effect reproduction rate.

In the final analysis of reproduction in these cultures, the pH change must be regarded as an additional variable. This variable might be eliminated by continuous automatic pH adjustment to a constant value; or the whole problem might be avoided by growing organisms in a chemostat. The latter has actually been accomplished in this laboratory with *Tetrahymena geleii*, and will be described in a later publication.

Summary

A continuous increase in the pH of a developing culture of *Tetrahymena geleii* was demonstrated. It was pointed out that this shift constitutes an additional parameter in reproduction rate experiments. Corrective measures were suggested.

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THEORETICAL FORMULAE FOR REPRODUCTION OF FREE-LIVING PROTISTA

IBEN BROWNING, LLOYD S. LOCKINGEN, AND WILLIAM J. WINGO*

An intensive effort has been made in these laboratories to accurately describe the reproductive behavior of the protozoan *Tetrahymena geleii*, in bacteria-free, liquid culture. Given a set of carefully standardized conditions (Browning, Brittain, and Bergendahl, this journal), accurate prediction of future populations at specific times was desired.

The mathematical description of the ideal case in which reproduction is not limited by any environmental factor was found to be identical with the classical first order chemical reaction formula. This formula with symbology used by this laboratory is as follows:

$$P_t = P_o e^{Kt}$$

(see any Physical Chemistry text), or

$$P_t = P_o \times 2^{t/tg}$$

where P_o = number of organisms (population) per ml. upon inoculation

P_t = number of organisms population per ml. at any time t

t = a given time

tg = time per generation

K = a constant.

This formula is valid only when there are no limiting factors. It will permit accurate prediction of subsequent populations only at very low values (see Figure I). When we used the second order chemical equation derived for biological use by McKendrick and Pai (1910–11), and applied to *B. coli* populations (see Figure I), the data obtained by our experimental methods is accurately described. This formula, altered to fit our symbols, is:

$$\frac{2.303}{P_x} \log_{10} \frac{P_t(P_x - P_o)}{P_o(P_x - P_t)} = Kt$$

*From the Department of Biology and Department of Biochemistry, the M. D. Anderson Hospital for Cancer Research, Houston, Texas. Received for publication August 15, 1952.

where P_x is the maximum population. (See Appendix I for general equations and necessary assumptions.) A useful extension of this formula for the purpose of predicting maximum population of a given culture is demonstrably workable and surprisingly accurate. This equation is:

$$P_x = \frac{P_1P_3^2 - 2P_1P_2P_3 + P_2^2P_3}{P_2^2 - P_1P_3}$$

where P_1, P_2, and P_3 are three data points determined by population counts; and where the times are equally spaced ($t_2 - t_1 = t_3 - t_2$). The more general equation is discussed in section II in the Appendix.

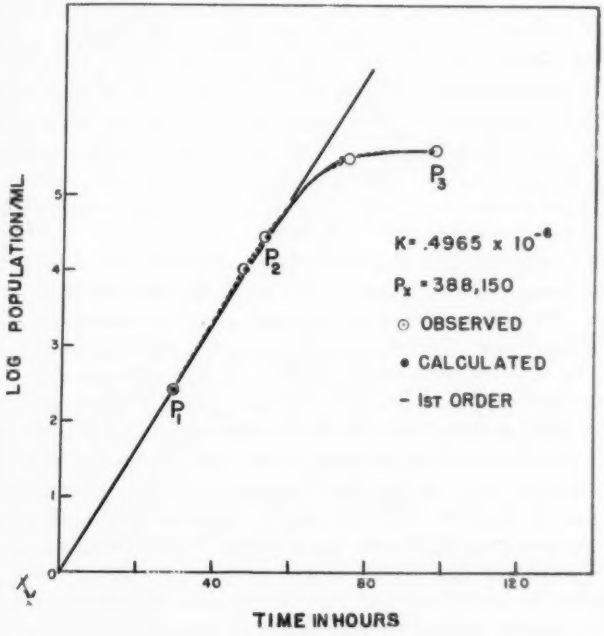

FIGURE I

Under different culturing conditions, such as unusual (low) oxygen concentrations, intermediate or higher order chemical

reaction equations (see Appendix II) describe the reproductive behavior of *Tetrahymena geleii* more accurately than the second order equation shown above.

Discussion

A set of equations has been derived on the basis of a series of assumptions. The inadequacy (but not necessarily inaccuracy) of these is pointed out, yet despite their shortcomings, they lead to useful formulae. These fit with considerable accuracy all population data obtained under our various conditions. Credibility is thus reflected on the original assumptions of simple autocatalysis.

The use of these formulae in conjunction with a linear regression computation, enables the worker to state with considerable accuracy a single number, K, which characterizes a whole set of population data on a single growth experiment. Under rigidly maintained, comparable conditions, a set of experiments may be characterized by a mean K plus or minus the standard error. Such figures permit application of standard statistical tests of significance. Once a population count is made, no further subjective treatment of data is necessary, provided these processes are adhered to.

It is obvious that the derivations are based on idealized assumptions, and that other factors exert an effect in actual processes. Variation in time of cleavage after assimilative collision (see Browning, Brittain, and Bergendahl, this journal), variation in pH (see Browning and Brittain, this journal) and either additional and/or metabolic limiters produce deviations from these ideal assumptions. The fundamental nature (and the probable accuracy) of the formulae is reflected by the nearness of fit to data, not only on chemical reactions, *B. coli*, and our organisms, but also fish (Volterra, 1928), human babies (Robertson, 1923) and passenger auto registration, U. S. (Ridenour, 1951). It is expected that the formulae will be altered, extended and further perfected to take into account more subtle aspects of environment and division behavior of *Tetrahymena geleii*.

Appendix I

The following assumptions are made in order to formulate the simplest conceivable reproduction formulae:

(a) That reproduction depends only upon the acquisition of particles (molecules or molecule aggregates) by organisms;

(b) That of these particles, only one species is in sufficiently short supply to be limiting;

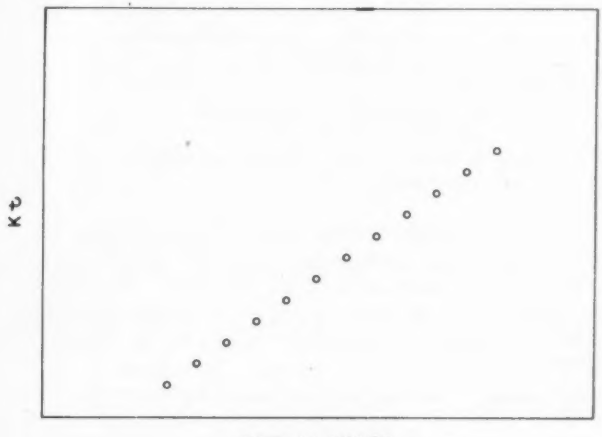

FIGURE II

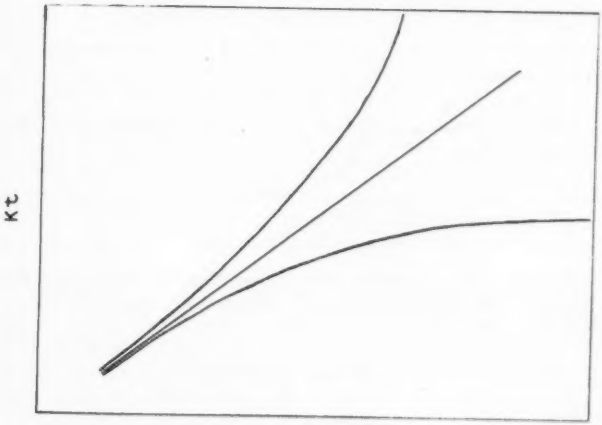

FIGURE III

(c) That these "factor" particles are required for reproduction only, and thus the requirement is proportional to number of organisms produced;

(d) That, given a fixed number of particles required for each reproduction, the standard rules of kinetics obtain.

If we adopt the symbols:

P_o = population per ml. at time = 0, or upon inoculation,
P_t = population per ml. at any time = t,
P_x = final population,
F_o = "factor" particles per ml. in fresh medium,
F_t = "factor" particles per ml. at time = t,
n = number of "factor" particles required per division,
K = reproduction constant,
t = time,
a = number of "factor" particles required for reproduction,

then a number of basic equations can be expressed. It should be understood that the symbol *F* denotes *only* "a limiting factor." No effort has been made to analyze or postulate the species of limiter, nor is there any intended implication that the limiters are the same in different orders of reactions.

These boundary conditions will hold under our stated conditions:

$$F_o = F_t \text{ when } t = 0.$$

When

$$F_t = 0, P_t = P_x \text{ and } t = \infty.$$

Assumption (c) above can be expressed in this manner:

$$F_o - F_t = a(P_t - P_o),$$

thence

$$F_t = F_o - a(P_t - P_o).$$

A combination of the assumptions a, b, c, and d produces the following kinetics expression:

$$\frac{dP_t}{dt} = KP_tF_t^a$$

$$= KP_t[F_o - a(P_t - P_o)]^a.$$

(See any Physical Chemistry text.) Integrating this expression between its limits, we can obtain the formula for any given value of *a*.

With a = 0, 1st order

$$2.303 \log \frac{P_t}{P_o} = K_1t;$$

with a = 1, 2nd order

$$\frac{2.303}{P_x} \log \frac{P_t(P_x - P_o)}{P_o(P_x - P_t)} = K_2t;$$

with a = 2, 3rd order

$$\frac{1}{4P_x^2}\left[2.303\log\frac{P_t(P_x-P_o)}{P_o(P_x-P_t)}-\frac{P_x}{P_x-P_o}+\frac{P_x}{P_x-P_t}\right]=K_3t;$$

with a = 3, 4th order

$$\frac{1}{P_x^3}\left[2.303\log\frac{P_t(P_x-P_o)}{P_o(P_x-P_t)}-\frac{1}{2}\left(\frac{2P_x-P_o}{P_x-P_o}\right)^2+\frac{1}{2}\left(\frac{2P_x-P_t}{P_x-P_t}\right)^2\right]=K_4t;$$

with a = 4, 5th order

$$\frac{1}{16P_x}\left[\frac{2.303}{16P_x^3}\log\frac{P_t(P_x-P_o)}{P_o(P_x-P_t)}+\frac{1}{3(P_x-P_t)^3}-\frac{1}{3(P_x-P_o)^3}\right.$$
$$\left.+\frac{(2P_x+P_t)^2}{32P_x(P_x+P_t)^2}-\frac{(2P_x+P_o)^2}{32P_x(P_x+P_o)^2}\right]=K_5t.$$

Interpretation of data by use of the above formulae is, at best, laborious. If the population data be expressed as number of cells per unit volume, then a graph of log of numbers against time will give a standard reference type of curve (see observed data, Figure I). Some experience will enable the worker to estimate the order of that growth formula which will apply. If a simple second order curve is apparently applicable, the maximum population may be calculated by taking three (or four) data points (see Appendix II). If one assumes that $P_o = 1$ for simplicity (and attempts to hold the experimental inoculum close to this figure for the sake of accuracy), simple substitution into the second order formula will yield a *Kt* solution corresponding to each population count (P_t) inserted. Plot *Kt* (calculated) against observed time (t) of sampling, and a straight line plot should result (see Figure II). If the plotted points curve downward (see Figure III), the reproduction rate corresponded to a higher order reproduction rate. The converse is true if the plot curves up. If the plot be a straight line, then *K* is a constant in the *Kt* solutions of population data. If the maximum population (P_x) is properly calculated, the last data point should be in a straight line with previous points. If the last point be high, the calculated maximum population is too low, and another estimate is in order.

This exceedingly laborious method will permit calculation of maximum populations of any digital order reproduction formula by the process of successive approximations.

If the plot of *Kt* is extended from *0* to its numerical value, backward extrapolation of the straight line data to the point of intercept with the *0* value locates that theoretical time at which there was one organism per millimeter. Such backward extrapolation may be more accurately accomplished statistically, using standard linear regression (Snedecor, 1946),

provided that all data points be regarded as equally significant. Given this calculated 0 time, a correction may be applied to all observed times, and "corrected time" obtained. Kt divided by "corrected time" yields the value of K.

Appendix II

The calculation of the maximum population, P_x.

The substitution of specific data points (population per milliliter at time of P_1, t_1; P_2, t_2; P_3, t_3), into the second order formula yields

$$\frac{2.303}{P_x}\log_{10}\frac{P_2(P_x - P_1)}{P_1(P_x - P_2)} = K(t_2 - t_1) \qquad (a)$$

$$\frac{2.303}{P_x}\log_{10}\frac{P_3(P_x - P_2)}{P_2(P_x - P_3)} = K(t_3 - t_2) \qquad (b)$$

Division of (a) by (b), and rearrangement, gives:

$$\log_{10} P_2 - \log_{10} P_1 - \left(\frac{t_2 - t_1}{t_3 - t_2}\right)\log_{10} P_3 + \left(\frac{t_2 - t_1}{t_3 - t_2}\right)\log_{10} P_2$$

$$+ \log_{10}(P_x - P_1) - \log_{10}(P_x - P_2) = \left(\frac{t_2 - t_1}{t_3 - t_2}\right)[\log_{10}(P_x - P_2)$$

$$- \log_{10}(P_x - P_3)]$$

which permits solution for P_x by the process of successive approximations. If the reproduction of *Tetrahymena geleii* were precisely according to the initial equation, the solution for maximum population, P_x, would yield an identical result regardless of the data points used, modified by initial sampling accuracy. Such is not the case, however. The fact is that the predicted final population increases with the highest data point picked. The above formulae simply force the calculated curve to fit the three points used—and could be forced to fit four points, instead.

For practical purposes of obtaining the nearest fit of theoretical curve to data points, points corresponding to P_1, P_2, P_3, on the curve of observed data in Figure I should be chosen. If such points are equally spaced in time, the simplification solution shown in the main text of this paper results from the above division.

One reason for the progressive change in theoretically predicted maximum population is contained in the paper in this journal by Browning, Brittain, and Bergendahl.

The fact that the time between "assimilative collision" and fission increases with the age of the culture, causes the rate of multiplication at any time toward the end of the growth period actually to reflect the collision rate at an earlier time (this lag increases as the culture ages). As a consequence, during the entire "negative growth phase" (Phelps, 1935), the actual reproductive rate is progressively higher than predicted

on the basis of the implicit assumption in Appendix I that division is instantaneous upon "assimilative collision."

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CELLULAR REPRODUCTION EFFICIENCY IN VARIOUS OXYGEN CONCENTRATIONS

IBEN BROWNING, JANET C. BERGENDAHL, AND MADELEINE S. BRITTAIN*

There have been numerous studies on the relationship of oxygen to "growth" in various organisms. In this laboratory, oxygen was found to be an important factor in the determination of reproduction rate of *Tetrahymena geleii;* however, a search of the literature failed to give a clear picture of its effect. Hall (1933) found *Colpidium campylum* (later identified as *Tetrahymena geleii*) has a behavior comparable to that of the facultative anaerobes among the bacteria. He also found that in a peptone medium under anaerobic conditions "growth" was approximately 50% of that under areobic conditions. On the other hand, Pace and Ireland (1945) found that *Tetrahymena geleii* fails to grow with an oxygen pressure of less than ten millimeters of mercury, whereas the optimum pressure is five hundred millimeters. The criterion of growth in the case of Pace and Ireland is the increase of organisms from the inoculum of $2{,}000 \pm 200$ organisms to the number found after four days with no intermediate determinations. Their medium was 2% Proteose-Peptone.

Preliminary studies in this laboratory had shown that the concentration of oxygen in a gas which is bubbled through a protozoan culture constitutes a major parameter in the control of reproduction rate and determination of final population. The following series of experiments was set up to measure the effect of oxygen concentration upon reproduction.

Materials and Methods

The organism used in this study was the ciliated protozoan *Tetrahymena geleii* (Thomas-Phelps strain) cultured under the conditions described in the next article in this journal. The medium used in all of the experiments for which data are given below was made up in a single batch and was distributed into

*From the Department of Biology, the M. D. Anderson Hospital for Cancer Research, Houston, Texas. Received for publication August 15, 1952.

culture flasks described in the previously mentioned article. Aliquot counts were made in Sedgwick-Rafter counting cells according to standardized procedures. Four flasks each were bubbled with 1, 5, 20, 50 and 99.5% oxygen, respectively. The complementary gas in each case was nitrogen, which is an inert gas so far as these organisms are concerned. The gas mixtures used are commercial preparations and are accurate to 0.1% in their composition.

Data and Results

The data were processed according to the formulae contained in the paper by Browning, Lockengen, and Wingo in this journal. Each set of data obtained from a single culture flask was analyzed for three features: final population, maximum reproduction rate (minimum population doubling time), and order of growth response. All proven contaminated cultures were thrown out. These data are listed in Table 1 giving averages at different oxygen concentrations.

TABLE 1

I Percent Oxygen Concentration	II Final Population Per Ml.	III Minimum Population Doubling Time	IV Order of Reaction	V Number of Experiments
1	184,600	4.87	3½	4
5	319,600	3.68	3+	3
20	287,600	3.45	2	3
50	192,600	4.22	2	3
100	67,200	6.43	2	3

In analyzing these data, no account is taken of the pH shift (see Browning and Brittain, this journal) and its effect upon reproduction rate (Wingo and Anderson, 1951). The effect is real but small, and consequently these calculations are not entirely accurate.

Conclusion

From the data in Table 1 and Figure I, we can conclude that in this medium the optimum oxygen concentration for reproduction is in the range of 5–20%, as this range of oxygen concentration permits the maximum final population and the maximum reproduction rate.

The data demonstrate a striking relationship between final population and reproduction rate. In general, the more organisms that can be produced under our conditions, the more rapid is the rate of reproduction during the early phase of culture development.

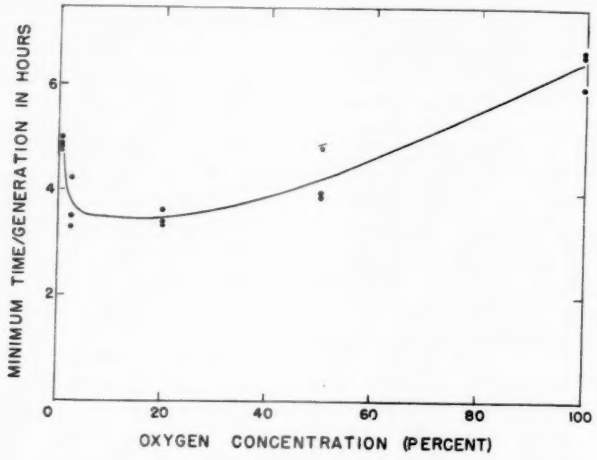

FIGURE I

At oxygen concentrations higher than optimum, the reaction order of growth remains constant and is most accurately described as having a single limiter. The decrease of both reproductive rate and final population reflects the increasing destruction of the limiter by increased oxygen concentrations. Those cultures growing in oxygen concentrations lower than optimum demonstrate reaction orders higher than two. This indicates that there is more than one limiter per reproduction. Such a change from one to more than one limiter suggests a shift of metabolic processes with a change of oxygen pressure. The associated decrease of final population could be due to a transfer of limiting action from one material to another. It is undoubtedly true that culture development in an unbubbled culture is accompanied by a continuous decrease of dissolved oxygen. Since replacement is by diffusion through the air-medium surface, an oxygen gradient is produced due to the requirements

by the organisms. Thus in an unbubbled culture, an indeterminate gradient plus a continuous shift of average oxygen concentration individually and together alter reproduction rate and final population expectancy. Such a culture has a reproduction characteristic which discourages, or perhaps defies, analysis.

The method of interpretation of results made in this study is a very accurate criterion of effect. It should be possible to measure other limiters and limit effectors by this interpretative method with considerably more sensitivity than by methods previously used.

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SYNCHRONOUS AND RHYTHMIC REPRODUCTION OF PROTOZOA FOLLOWING INOCULATION

IBEN BROWNING, MADELEINE S. BRITTAIN, AND JANET C. BERGENDAHL*

Synchronous and/or rhythmic cell division has been reported in various species. A prime example of this phenomenon is the case of early cell division in an amphibian zygote. Likewise, the cells near the periphery in animal-cell tissue culture were reported dividing rhythmically by Bauer (1941). The cells which constitute a *Lilium longiflorum* anther are known to divide synchronously (Erickson, 1948). Many other examples may be found in organized tissues where cells are in position to influence each other.

As a result of growth experiments on protozoa in pure culture (see Hall, 1941, for a review), the phenomenon of allelocatalysis so convincingly espoused by Robertson (1923) has been laid to rest. In the light of this body of literature one must conclude that any reproductive rhythmicity in a protozoan culture is not a result of mutual influence. Three causes of reproductive synchronism are: (1) an oscillating population balance with another—or contaminating organism (Volterra, 1928); (2) a rhythmic environmental influence such as a diurnal change in light or heat; or (3) a reproductive oscillation following a single environmental stimulus.

Consideration of the theoretical mathematical formulae for reproduction of protozoa (see Browning, Lockingen and Wingo, this journal) leads to the prediction that a "burst" of reproductive activity should occur immediately subsequent to inoculation. This series of experiments was designed to test the prediction, thence by inference the theory.

Materials and Methods

Organisms were grown in the following medium:

NaCl	0.0145 gm.
KCl	0.0020 gm.
KH_2PO_4	0.0453 gm.
$MgSO_4$	0.0124 gm.
Ca Gluconate	0.0049 gm.
Proteose-Peptone	10.0000 gm.

*From the Department of Biology, the M. D. Anderson Hospital for Cancer Research, Houston, Texas. With the technical assistance of the Department of Photography. Received for publication August 15, 1952.

Dissolve in several hundred milliliters of triple-distilled water. Adjust with 0.5 N NaOH to pH 6.8. Add triple-distilled water to a volume of 1,000 ml. Filter and add 1 drop of oleic acid.

This medium is modeled after that of Phelps (1935), but has the advantage that none of the components is deliquescent.

A growth flask was especially designed for this experiment, and has proved itself to be ideal for many other similar experiments.[1] The design is shown in Figure I. In normal operation the bubbling tube is plugged with cotton and the neck is sealed with a rubber stopper and covered with aluminum foil.

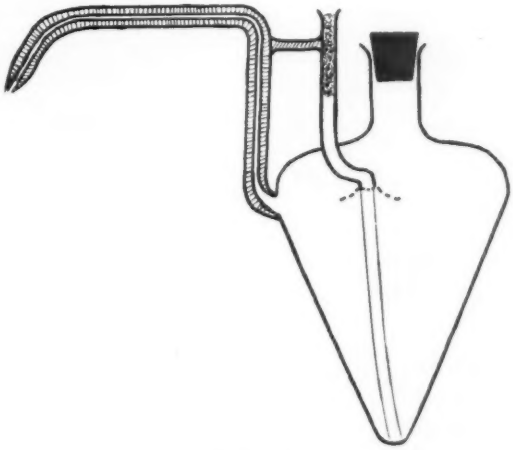

FIGURE I

From a single large batch of medium, a seventy-five milliliter aliquot was placed in each growth flask which was to be used for culturing organisms. Inoculation from a carefully standardized[2] control culture was made with a very small platinum bacteriologists' loop and the culture was permitted to develop in a dark, thermostatted water-bath at 28.3° C. Water-saturated air

[1] Flasks of this design may be obtained from The Houston Glass Fabricating Co.

[2] These organisms have been maintained for two years in standard medium at approximately constant temperature with weekly transfers.

was bubbled slowly through the culture and one or two population counts were made daily using a standard Sedgwick-Rafter counting chamber with a dark-field, 100X microscope. The "experiment flask" contained approximately twenty milliliters of sterile medium from the same batch as the inoculum source culture. Medium in this flask was adjusted to the same pH as the culture and was put under the same conditions. At each predetermined stage of culture development (see Figure II,

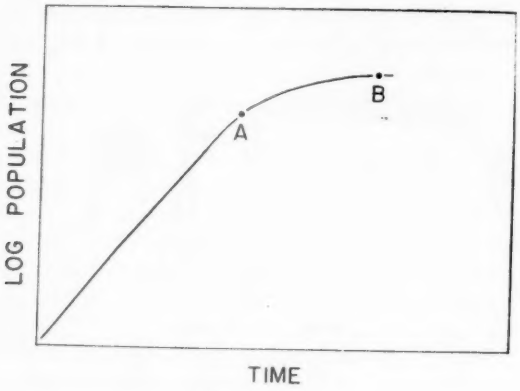

FIGURE II

Points A and B), a large aliquot of Tetrahymena culture was transferred aseptically to an experiment flask such that there were approximately 60,000 organisms per milliliter of medium. A sample was taken immediately and was fixed with 5% formalin. As soon as the organisms settled to the bottom of their Sedgwick-Rafter container, three random-sample fields were photographed in bright contrast. At the end of each subsequent 10 minute period for seven and one-half hours, a similar series of pictures was taken .This particlar experiment is simply an elaboration of four preliminary experiments which gave essentially the same results. As a guide, a table of division figures (see Figure III) was taken from Fergason (1940), and was used in making differential counts from the projected negatives. In all cases, three people concurred on the classifications of division stages of the photographed organisms.

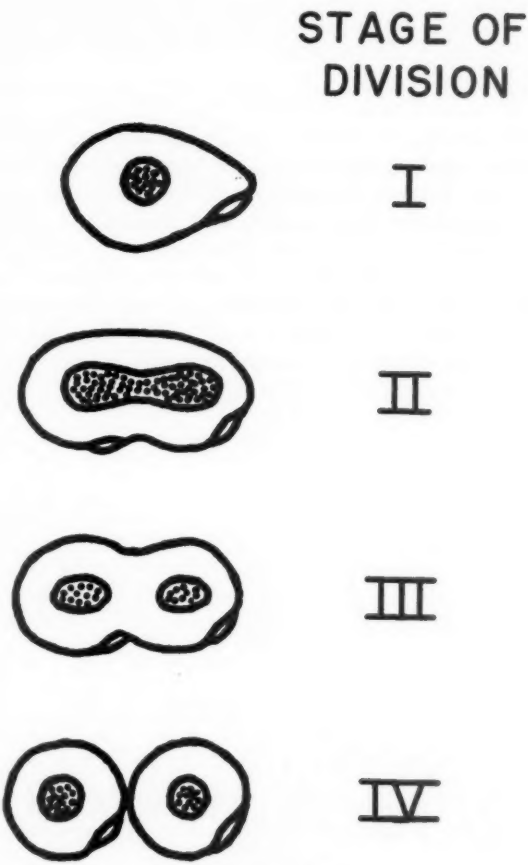

FIGURE III

Data and Results

Cells were counted and classified without selection so that the smallest sample considered was considerably in excess of 100. Figures IV and V show the results of the computed percentage of division figures. It should be noted that Stage 2 organisms may be distinguished only in silhouette, therefore, figures VI

and VII show the most reliable data. In these figures, the total percent of Stages 3 and 4 is shown plotted against time.

Discussion

A very significant "burst" of division occurs in the culture of protozoa following inoculation of a large population into fresh medium. The percent of organisms in division increases rapidly to a maximum, then drops sharply to a minimum; subsequently (as in Figures IV and VI), it increases to a secondary maximum and drops again. Such a synchronous division must be attributed to a single environmental stimulus. Figures IV and V show that Stages 2, 3, and 4 reach peak values successively. Such a sequence is to be expected and bears out the validity of the data.

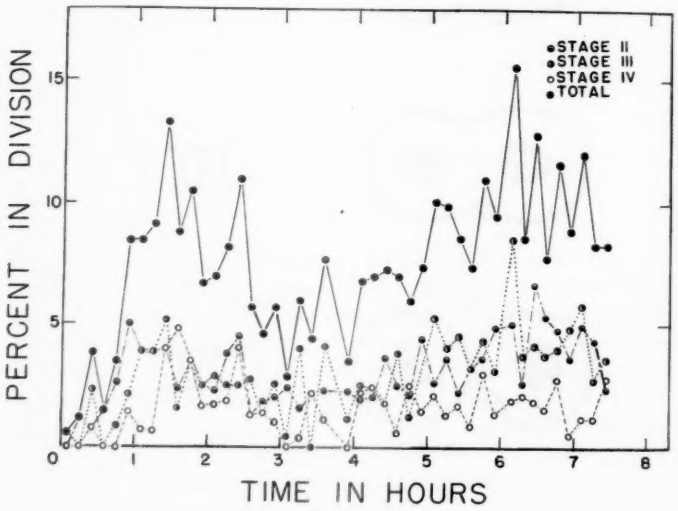

FIGURE IV

Previous work in this laboratory (Browning, Swinford and Varnedoe, 1952) has shown that a constant length of time is required for fission to occur independent of the generation time. The present work indicates that organisms taken from a culture

at Points A or B, respectively, are metabolically equally ready to divide (see, also, Wingo and Cameron, this journal), but lack some limiting substance which is obtained immediately following inoculation or very shortly thereafter. This hypothetical substance permits the cells to proceed with division. In Case B there is a lag of some three hours before the majority of the cells indicate the beginning of cytoplasmic fission. The great increase in numbers of cells in division after three hours indicated (see Figure V and Figure VII) that most of the material which was limiting was acquired by the majority of the organisms within a span of two hours after inoculation. If this is the case, then we may infer that a lapse of some three hours occurs between assimilation of the limiting factor and the consequent cell division.

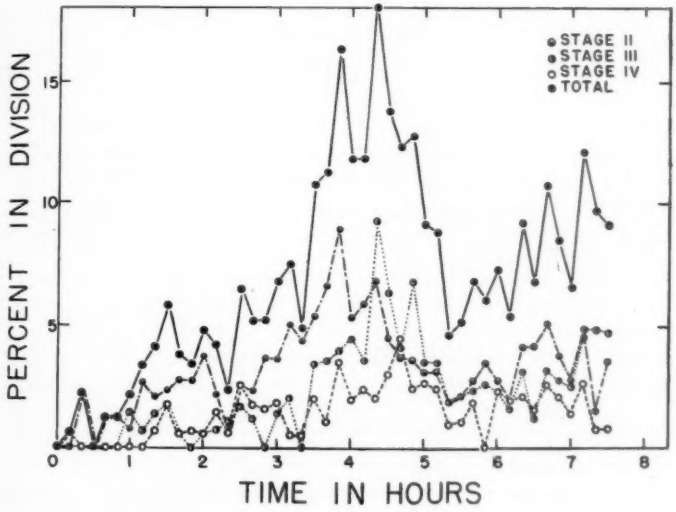

FIGURE V

If one were to assume that all organisms had divided at the end of the first cycle (see Figures IV, V, VI, or VII), one could then compute the length of time required for an organism to undergo complete fission once it has started visibly into Stage 2. Such a computation would take the form of integrating with a

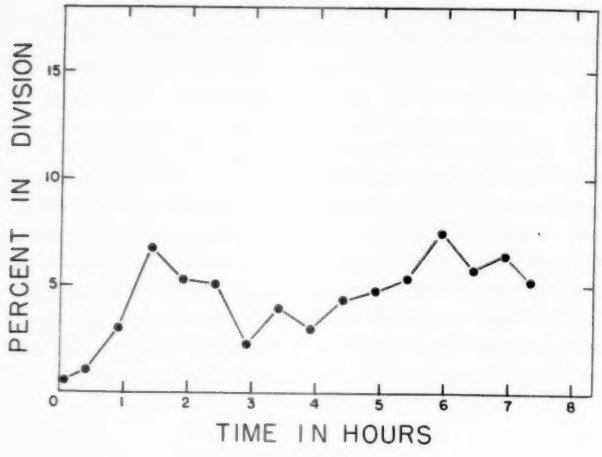

FIGURE VI

planimeter the area under the curve, dividing the area by an appropriate factor and reducing the quotient to minutes. The results of such computations for both Points A and B are compared with similar information previously obtained (Browning, Varnedoe, and Swinford, 1952) by two different methods: computation and observation.

TABLE 1

Time of Division in Minutes

Stage of Division	I Computed	II Point A	III Point B	IV Observed
II	7	5.1	10.4	15–
III and IV	6.8	7.1	11.1	12.5

In view of the fact that the computed values shown in Column I are for populations no higher than approximately three-fifths the final population, the values would correspond most closely to Point A (Column II.) Column III shows a corresponding set of figures for Point B, which is later time in the culture development. Point IV is not strictly comparable since it was taken under different conditions (see Browning, Varnedoe, and Swin-

ford, 1952). Such corresponding figures concerning time of fission reflect accurately on the original assumption and on the data as a whole.

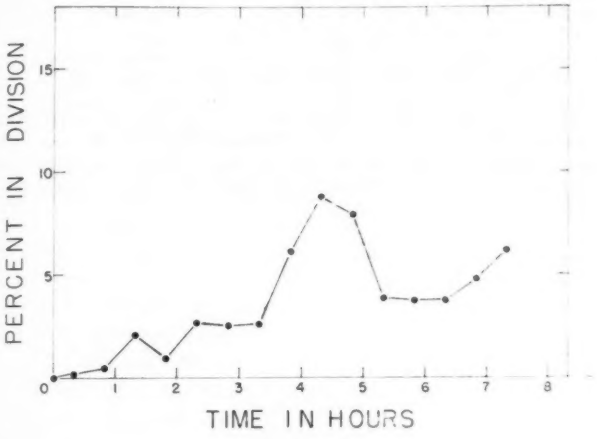

FIGURE VII

We may draw a very strong inference from these data that *Tetrahymena geleii* under these conditions are limited in reproduction by a particular "factor." A state of complete metabolic preparedness for division is "normal." The final population (essentially reached at Point B, Figure II) is not a function of metabolic considerations; rather it is a function of exhaustion of the limiting "factor."

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STUDIES ON TOXIC PLANTS FOR ANTIBACTERIAL EFFECTS

HENRY M. BURLAGE, MARY ELIZABETH JONES, GEORGE F. MCKENNA, AND ALFRED TAYLOR*

In recent years a great deal of emphasis has been placed on the search for chemotherapeutic agents active against bacteria, fungi, and viruses. In the case of the bacteria there has been notable success; in the case of the viruses an opening wedge is seen in such agents as chloromycetin, while the fungi remain almost untouched by antibiotics. The number of these agents that have been reported is quite numerous (Baron, 1950); however, only a few have withstood rigorous testing procedures to become tools of the clinician. Those in the greatest use are from fungi, particularly the actinomycetes and molds. The number of studies on these groups of organisms with reference to production of antibiotic agents is great and continues to increase, because of their potentialities.

The vast amount of work along this line has focused attention on the possibilities latent in other materials of a biological source. Although some work has been done on various plants with regard to antibacterial effect, (Atkinson and Ramsford, 1946; Atkinson, 1946; Cavallito and Bailey, 1950–51; Hatfield, 1948; Huddleson, Du Froin, Barrows, and Giefel, 1944; Little and Grubaugh, 1946; Lucas and Lewis, 1944; Lucas, 1948; Lucas *et al.*, 1951; Osborn, 1943; Robertson *et al.*, 1951; Seegal and Holden, 1945; Southam, 1946) only a few systematic studies have been conducted to survey the possible effects of a number of plants on a fairly wide range of bacterial organisms.

Within the last few years a project has been started at The University of Texas with the dual purpose of screening extracts of various plants (Taylor *et al.*, 1952), particularly those occurring locally and that have been described as being toxic to animals on investigation, with regard to their effect on growth of tumor, bacteria, and viruses. Insofar as the antibacterial work

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is concerned, 200 individual tests have been made on plant extracts and of this number 53 extracts have shown to have some degree of antibacterial effect. This includes at least 12 plants, four of which have shown unusual antibacterial activity and are receiving special attention.

This type of work presents certain problems not encountered to such a degree in studies of materials having as their source microorganisms. Principal among these is the seasonal variation of constituents of plants used, some of which are available only during the spring, summer, and early fall. Of these, variations in composition, both quantitative and qualitative, may occur. There may also be differences due to soils in which they grow, whereas the media used in the growth of microorganisms is a controllable factor. Growth factors as a whole are subject to variation since the nature of such a survey makes it necessary to use many plants as they are found in nature. It is also difficult to draw a conclusion from comparison of antibacterial activity of a material which is by nature a mixture of constituents with a chemically pure commercial antibiotic, especially on a direct weight basis.

Careful technics described in a previous paper (Taylor *et al.*, 1952), are employed in preparation of the extracts in order to obtain and retain, as far as possible, the active constituents in unaltered form (Taylor *et al.*, 1952). Since the nature and amounts of these constituents are not known in the original plant, it follows that concentrations of the same can be known only in reference to weight of plant material to volume of solvent. Freeze-drying of the plant material under high vacuum refines this to a degree, but this process is usually carried out after the original screening indicates some activity.

This entire process of extraction is accomplished at temperature as low as is practicable with maceration after comminution by means of a mortar and pestle or Waring blender. The extracts used in antibacterial testing were generally prepared with the following solvents: water with a pH of 6.6, 7.0 and 7.4, respectively, boiling water at pH 7.0, absolute alcohol, and acetone. In the case of the last two, the solvents were evaporated off, and the actual portion used for testing is the water soluble (at pH 7.0) portion of the residue. Sterilization is ordinarily done by

filtration and in some cases by autoclaving at 15 lbs. for 15 minutes. The former treatment is the general practice in order to avoid possible alteration of constituents in the extract, but if hot aqueous extract shows promise, this latter technic of sterilization is tried.

Each extract so prepared is tested as soon as possible after its preparation since, in some cases, radical changes in action have been noticed after a short period of storage even though the extract in question has been frozen during this time.

In vitro testing for sterility of the extracts is accomplished by transferring a few drops of the extract by means of a pipette into tubes of semi-solid brain-heart infusion which is then incubated for 24–48 hours at 37° C. This was chosen as the medium of choice because of its ability to support a wide range of organisms including aerobes, microaerophiles, and anaerobes. Since many anaerobes, particularly sporeformers, occur in the soil and might be expected to be on the surface of plant material, this is of particular advantage.

Due to the fact that the main purpose of these tests is to screen extracts of the various plants for antibacterial effect the tests used are designed to ascertain evidence of activity, even though it may be relatively weak. Since concentration of active material may be almost negligible, such results may be of considerable interest.

Five tests are employed, four of which are *in vitro*, and one of which is carried out with chick embryos. Of the *in vitro* tests, three are done by plating technics and one employs turbidimetry as well as plating.

Of the plating methods the streak method (Anigstein *et al.*, 1950; Randall *et al.*, 1945) is used only as a rough screening process, the filter paper disc modification of the Oxford Cup (Vincent and Vincent, 1944) method being the means of measurement of inhibition of choice among the plate technics (Loo *et al.*, 1945). The Oxford Cup method (Foster and Woodruff, 1944) was used for comparison with the filter paper disc method for many of the extracts but was dropped from usual procedure with the addition of turbidimetric methods.

A series of tests to determine the antibiotic sensitivities of the test organisms was also done by both cup and disc methods. The

sensitivities of the organisms in regard to the photometric technic was studied in comparison to degree of inhibition of turbidity by running tests of penicillin, streptomycin, terramycin and aureomycin by this method since these are most commonly used for comparative purposes with the organisms of the test list.

It is realized that in the interest of accuracy these sensitivities should be repeated at much closer intervals than is possible with present facilities and that these values are suitable only for estimate, having been done primarily to determine the relative degree of antibiotic sensitivity of the strains used under the conditions which they are maintained.

In the turbidimetric method the major problem is dilution of the extract, however the diffusion factor encountered in plating is greatly reduced by this method. This procedure is done in duplicate so that one series can be plated in order that the photometer (Klett) readings can be compared with the number of living organisms remaining. In quite a few instances far greater inhibition has been shown by comparison of plate counts of tests vs. plate counts of control than by the same comparison by turbidimetry (see Table 1), the latter being greater, due to inclusion of dead organisms. The turbidimetric method is recognized as one of the standard methods in the field of assay work in which growth of mircroorganisms is to be compared. Since the typical action of an antibiotic substance is not the killing of the organisms against which it acts but inhibition of their growth beyond the original inoculum, this method is generally satisfactory. Further study is necessary to determine what factors are responsible for the fact that in several cases in this work a higher degree of inhibition was detected by plate count than by turbidimetry when the cultures were identical insofar as possible originally.

A relatively simple set-up is employed for the turbidimetric method in that for each organism tested four tubes are inoculated, a control of 1.0 ml. nutrient broth, a 1:10 dilution of 0.1 ml. extract in 0.9 nutrient broth, at 1:5 dilution of 0.2 ml. extract to 0.8 ml. nutrient broth, and a 3 to 7 dilution of 0.3 ml. in 0.7 ml. nutrient broth. All have a total volume of 1.0 ml. and an inoculum of 0.005 ml. of 20 hour culture of the respective organism incubated at 37° C. The entire set is then incubated 18–24 hours at 37° C., after which one set is plated out for

counting, and the other is read for degree of turbidity using a Klett-Summerson Colorimeter (Summerson, 1939), that has been standardized by use of a tube of sterile nutrient broth from the same batch as that used to incubate the organisms.

TABLE 1

Comparison of Klett Turbidimetric Readings vs. Plate Counts Where Large Numbers of Dead Organisms Are Present

Extract	Control	0.1 ml. extract	0.2 ml. extract	0.3 ml. extract
Klett Readings				
Staphylococcus aureus	43	29	56	95
Escherichia coli	58	43	50	90
Bacillus subtilis	45	34	50	110
Neisseria catarrhalis	44	44	50	104
Shigella dysenteriae	38	34	48	54
Plate Counts				
Staphylococcus aureus	Confluent growth	Some reduction	Discrete colonies	Approx. 2,000/ml.
Escherichia coli	Confluent growth	Some reduction	Discrete colonies	Approx. 1,100/ml.
Bacillus subtilis	Confluent growth	13 colonies	1,500/ml.	Discrete colonies
Neisseria catarrhalis	Confluent growth	No growth	No growth	1,500 colonies/ml.
Shigella dysenteriae	Confluent growth	5,000/ml.	5,000/ml.	4,000 colonies/ml.

NOTE: These results indicate that the Klett readings were influenced greatly by the presence of dead organisms since each colony represents one living organism in the original material. It is also indicated that the lower concentration of extract is far more effective. Further study is being done in regard to suitable concentrations.

In vivo testing has been carried out if the results of the *in vitro* tests are generally favorable and is accomplished by use of the chick embryo technic (Taylor *et al.*, 1952), in which the test organism is injected into the yolk sac of the embryo and allowed to incubate at 99° F. for one hour. At the end of this period the extract or known antibacterial agent is injected into the membrane of the embryo. Controls do not receive the antibacterial agent but are injected with sterile water or physiological saline solution. It is then possible to compare the protection given the embryo by a given extract to that accomplished by a standard antibiotic.

Organisms against which the extracts were tested cover a wide range with regard to usual source. The following list of organisms used indicates their most common source:

Micrococcus pyogenes var. aureus FDA 209 (Staphylococcus aureus)—Human skin, boils, carbuncles, and abscesses.

Escherichia coli ATC No. 9,637—Intestines of man and animals, and urinary tract of man.

Proteus vulgaris ATC No. 9,623—Intestines of man and animals, and urinary tract of man.
Bacillus subtilis ATC No. 6,633—Soil.
Salmonella paratyphi ATC No. 9,444 *(Salmonella paratyphi A)*—Intestines of man and animals.
Salmonella schottmuelleri ATC No. 10,258 *(Salmonella paratyphi B)*—Intestines of man and animals.
Salmonella typhosa ATC No. 6,229—Intestines of man and animals.
Pseudomonas aeruginosa ATC No. 10,197—Intestines of man and animals, urinary tract of man.
Neisseria catarrhalis ATC No. 7,900—Nasal mucosa.
Shigella dysenteriae (shiga) ATC No. 9,665—Intestines of man.

Only two, *Micrococcus pyogenes* var. aureus FDA 209 (Staph. aureus) and Bacillus subtilis ATC 6,633, are gram positive, this being of interest since antibiotics in general clinical use at the present time have proven more effective against these than gram negative organisms. For this reason the majority of the organisms composing the test list is made up of gram negative forms.

Principal pathogens included are those of the Salmonella group and *Shigella dysenteriae*, members of the former being the causative agents in typhoid and paratyphoid fevers, and the latter being responsible for bacillary dysentery. Of the antibiotic agents having effect *in vitro* on these organisms, few are capable of reaching the site of infection in sufficient concentration to carry on reliable therapy. *Neisseria catarrhalis* is closely related to *N. gonorrheae* but is generally considered a nonpathogenic member of the ordinary respiratory flora. *Pseudomonas aeruginosa* and *Proteus vulgaris* have both been implicated in mixed infections, particularly of the human urinary tract and are noted for their ability to withstand treatment in such cases. *E. coli* is an inhabitant of the intestines of a wide range of animal forms and is, under normal conditions, considered nonpathogenic, but due to its survival ability a rather difficult organism to attack by means of chemotherapy. *B. subtilis* is a soil form of no particular pathogenic significance but is closely related to *B. anthracis* and is the only sporeformer among the organisms comprising the test list.

For maintenance of these cultures, nutrient agar of final pH of 7.2 ± 0.1 is used except in the case of *Neisseria catarrhalis* which is carried in semi-solid brain-heart infusion.

Buffalo Bur (*Solanum rostratum* Dunal.) (See Table 2).

Extracts of this plant have so far shown only limited effect except against *Pseudomonas aeruginosa.* Further tests are in progress on freshly prepared extracts of spring growth.

Bull Nettle—(*Cnidoscolus texanus* (Muell. Arg.) Small) (See Table 2).

Extracts of this plant showed negative results.

Castor Bean (*Ricinus communis* L.) (See Table 2).

Extracts of this plant, unless heat-treated, contain ricin which is so toxic in nature as to make impractical the use of such an extract. An earlier extract which had been boiled gave moderate effect against *Staphylococcus aureus, Bacillus subtilis, Neisseria catarrhalis,* and *Shigella dysenteriae* which was roughly comparable to the effect of the ricin-containing extract thus indicating that an inhibitor other than ricin may be present.

Inky Mushroom (*Coprinus atramentarius* (Bull.) Fr.) (See Table 2).

Moderate effect has been observed in *in vitro* test of this plant, but further study is necessary to determine whether the effect was due to original material or breakdown products since the encouraging results were obtained on later tests and did not correlate with the early tests which were negative.

Eupatorium perfoliatum *(Urticaefolium)* (See Table 2).

Earlier tests indicated possible presence of an inhibitor, but repeat tests failed to reveal an effective principle.

Milkweed (*Asclepias decumbens* L.) (See Table 2).

Slightly acid extracts of this plant have shown some effect but do not show much promise as antibacterials.

Rain Lily (*Cooperia pedunculata* Herb.) (See Table 2).

Cooperia pedunculata Herb., *Cooperia drummondii* Herb. and *Zephyranthes texana* Herb. have been thoroughly tested, and in no case has there been more than moderate inhibitory effect.

Snow-on-the-Mountain (*Euphorbia marginata* Pursh.) (See Table 2).

Extracts gave no outstanding indications, although some effect was noted on the Salmonella tested.

TABLE 2

Results of Antibacterial Tests Zones of Inhibition in mm.*

Plant	Plant part pH of Solvent	Staphylococcus aureus	E. coli	Proteus vulgaris	Bacillus subtilis	Salmonella paratyphi A	Salmonella paratyphi B	Salmonella typhosa	Pseudomonas aerugi-nosa	Neisseria catarrhalis	Shigella dysenteriae
Inky Mushroom (Corprinus atramentárius) (Bull.) (Fr.)	Whole plant H_2O–pH 6.6	.....	.....	.....	.....	.....	slight	.....	1 mm.	.....	.....
Milkweed (Asclepiodora decumbens L.)	Bloom, leaves stems H_2O–pH 6.6	.....	.....	.....	.....	.....	.....	2 mm.	.....	.....	1 mm.
Milkweed (Asclepiodora decumbens L.)	Bloom, leaves stems H_2O–pH 7.0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Milkweed (Asclepiodora decumbens L.)	Bloom, leaves stems H_2O–pH 7.5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Bulb HCl–pH 6.6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Bulb and roots H_2O–pH 6.6	.....	.....	.....	slight	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Bulb, roots, stems, and blooms H_2O–pH 6.6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Leaves, and blooms H_2O–pH 6.6	.....	.....	slight	.....	.....	slight	.....	.....	.....	.....
Rain-Lily (Cooperia	Whole plant										

Rain-Lily (Cooperia drummondii Herb.)	Whole plant H_2O–pH 6.6	.....	.....	.....	.....	.....	.....	.....	1 mm.	.....	.....
Rain-Lily (Cooperia drummondii Herb.)	Bulbs and roots H_2O–pH 7.0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia drummondii Herb.)	Bulbs and roots H_2O–pH 6.6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Zephyranthes texana-Herb.)	Roots, bulbs leaves, stalk flower, & seed pod H_2O–pH 6.6	.....	.....	.....	.....	slight	.....	slight	slight	.....	.....
Rain-Lily (Zephyranthes texana-Herb.)	Roots, bulbs leaves NaOH–pH 7.5	.....	.....	.....	.....	slight	.....	slight	.....	.....	.....
Rain-Lily (Zephyranthes texana-Herb.)	Roots, bulbs, leaves H_2O–pH 7.0	.....	.....	.....	slight	.....	.....	.....	.....	.....	2 mm.
Rain-Lily (Zephyranthes texana-Herb.)	Bulbs, leaves seed pods Acetone*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Zephyranthes texana-Herb.)	Leaves, roots flower, stalk seed pod H_2O–pH 6.6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Zephyranthes texana-Herb.)	Roots, bulb leaves, stalk seed pod Acetone*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Lyophilized bulbs H_2O–pH 6.5	slight	slight	.....	.....	.....	slight	.....	.....	.....	.....

*Results shown here were obtained by Filter Paper Disc Method as cup method results correlated well.

TABLE 2 *(continued)*

Results of Antibacterial Tests Zones of Inhibition in mm.*

Plant	Plant part pH of Solvent	Staphylococcus aureus	E. coli	Proteus vulgaris	Bacillus subtilis	Salmonella paratyphi A	Salmonella paratyphi B	Salmonella typhosa	Pseudomonas aerugenosa	Neisseria catarrhalis	Shigella dysenteriae
Rain-Lily (Cooperia pedunculata Herb.)	Roots, bulbs, leaves H_2O–pH 6.5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Roots, bulb leaves, Ether*	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Roots, bulb leaves Chloroform H_2O–pH 6.68	.....	.....	.....	2 mm.	.....	.....	.....	.....	.....	.....
Rain-Lily (Cooperia pedunculata Herb.)	Roots, bulb H_2O–pH 6.45	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Buffalo Bur (Solanum rostratum Dunal.)	Leaves, flowers stem, seed pods young & old seeds H_2O–pH 7.0	.....	.....	.....	.....	.....	.....	.....	1 mm.	.....	.....
Bull Nettle (Cnidoscolus texana) (Muell. arg.)	Bloom, leaves stem. H_2O–pH 7.0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Castor Bean (Ricinus communis L.)	Seeds H_2O–pH 6.9 Extract autoclaved.	3 mm.	.....	.....	2 mm.	.....	.....	.....	.....	1 mm.	3 mm.
Castor Bean											

Castor Bean (Ricinus communis L.)	Seeds H_2O–pH 6.9	.....	.....	.....	1 mm.	.....	.....	.....	.....	1 mm.	3 mm.
Castor Bean (Ricinus communis L.)	Seeds H_2O–pH 6.5 Extract Boiled	slight	slight	.....	.....	.....	.....	.....	.....	.....	.....
Castor Bean (Ricinus communis L.)	Seeds H_2O–pH 7.0 Extract Boiled	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Eupatorium (Urticaefolium) (Reichard)	Bloom, stem roots. Absolute alcohol	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Snow-on-the-Mountain (Euphorbia marginata) (Pursh.)	Bloom, leaves stem, root NaOH–pH 7.6	slight	.....	.....	.....	.....	1 mm.	slight	slight	.....	.....
Snow-on-the-Mountain (Euphorbia marginata) (Pursh.)	Bloom, leaves stem, root Acetone	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Snow-on-the-Mountain (Euphorbia marginata) (Pursh.)	Bloom, leaves stem, root H_2O–pH 7.01	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Snow-on-the-Mountain (Euphorbia marginata) (Pursh.)	Bloom, leaves alcohol* stem, root Absolute	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

*Final solution is water soluble portion of ether, acetone, chloroform or absolute alcohol extract with solvent at pH 7.0.

As previously stated four other plants having given such promising results, they are being studied further and will be reported on at a later date.

Summary

In studying certain plants indigenous to Texas with regard to their effect on bacteria a system has been set up which is designed to meet the special problems of dealing with unstable plant material of unknown concentration and qualitative make-up. This consists of careful preparation of extracts, low temperature storage of these extracts both before and after sterilization, freeze-drying under high vacuum where desirable, and both *in vitro* and *in vivo* testing for antibacterial effects whenever the extract in question warrants both methods of study. The methods used afford maximum possibility of detection of any degree of activity that may be present under the conditions of testing.

On determination of antibacterial activity of an extract, wherever this effect is of significance, every effort is made to increase the concentration of plant material since the active principle may be only a minute percentage of the total composition of the plant. In more promising cases where chemical work is done, fats and sugars and other inactive constituents are removed to obtain as high a concentration of the effective portion as possible. Complete reports on chromatography are not as yet available, but this work is in progress on more active extracts found to date.

It is obvious that many factors inherent in such studies are of necessity outside the range of control. These include growth conditions of the wild plant, loss of enzyme action on storage, and destruction of active agents during extraction and storage. These factors make it most difficult to obtain repeatable results.

With all these conditions under consideration it is encouraging to note that a majority of the plants tested have shown some measure of antibacterial action, a few warranting more thorough investigation.

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OPPORTUNITIES IN HOSPITAL AIR-CONDITIONING*

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The material discussed here probably presents air-conditioning (AC) in somewhat different terms than most engineers are accustomed to thinking and dealing in. It is hoped that we will raise some questions for you to think about and perhaps stimulate you to help in answering them. We are going to be dealing here, not with BTU's or CFM's, but with BUGS in the AC equipment; not mechanical bugs, but living "bugs" better known as germs or organisms—organisms which can infect people and cause disease and death. We will consider the control of air-borne diseases, the possible role of AC in the spread or control of air-borne infections, especially in hospitals, and what could or should be done in hospital AC to ensure safe air.

When we consider how diseases and infections are spread and how man prevents their spread, we observe that man filters and chlorinates the water that he drinks. Sewage is treated by digestion processes and other methods, before final disposal. Food is refrigerated, preservatives added, canned under high temperatures and then cooked before being eaten. Milk is produced under hygienic conditions and then pasteurized to kill all pathogenic organisms. Insects which might carry diseases are eradicated with rotenone, DDT and other chemicals. People who have contagious diseases are quarantined for long periods and, besides, we are all careful whom we "contact" or with whom we go out. Where *air* is concerned, nothing in particular is done, for the most part, to control the possible transfer of disease-producing organisms.

The question arises whether air treatment should be considered necessary. A brief look at the average man shows that he breathes quite a bit of air each day. For instance, he breathes some 20 times per minute. Each breath approximates 1 pint (500 ml), or 10 liters per minute, 600 per hour or 14,400 liters per day (approx.). This is equivalent to almost 600 cubic feet of

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air per day. (Note: please check with slide rule.) From the physiological viewpoint, this air would have 12% oxygen and approximately 0.2% carbon dioxide. For comfort, the temperature should vary from 70–78° F. and humidity approximate 50% RH, both depending upon the work or activity of the individuals concerned. To prevent loss of appetite and energy which would occur from increase in odors (body) and/or high humidity, it has been suggested that 400 cubic feet per person and 1.5 air changes per hour or better be provided for the 10 CFM allowance for comfort. We now have the individual taken care of as to his physiological needs and comfort *but* what have we considered about the purity of the air as far as infectious organisms are concerned? Nothing! It brings to mind the analogy of asking the man who has just been seated in the electric chair if he is comfortable. We have provided for the individual's comfort, but he is due for a shock.

The shock, of course, is that this comfortable air that he is breathing could, and many times does, contain potentially dangerous disease-producing organisms. There are germs or organisms everywhere. Greater numbers are found in populated areas. Not all of these are dangerous. Of the fifty thousand or more different kinds that are known, only a few hundred are pathogenic or infectious for man. The air may contain only a few or many per cubic foot. How do organisms get into the air? Outside, the wind over soil or water picks up dust and water droplets containing organisms which become air-borne. Inversions of hot air rising over sun-heated areas also take organisms aloft. Many of these organisms survive the dessication and may travel as high as 35,000 feet and hundreds of miles with the wind. Also outdoors, but more important indoors, organisms may get into the air from people coughing, sneezing, spitting or even talking. Of importance indoors are the organisms from clothes and blankets, as well as the dust arising from sweeping or drafts of 50 CFM or more. Usually the large dust particles holding organisms such as bacteria or viruses settle out rapidly, but the smaller clumps or single organisms of one micron or less in diameter (one twenty-five thousandths of an inch) may stay suspended for hours or resuspend readily at the slightest motion. The number of organisms in indoor air varies with the number of people, amount of sneezing, coughing and talking, amount of

sweeping, motion and flow of air. Unfortunately, the number of pathogenic or disease-producing organisms increases with the number of people who congregate in a given area. Studies have shown that there are very few in open areas, but larger numbers in houses and offices and, of greater importance, even larger numbers in schools and hospitals, where we would like to have ideal conditions for children and for sick people.

If all this is true about the number of disease-producing organisms, then how does the body protect itself? Fortunately, the body has several lines of defense to protect itself against the germs which surround it. The design of the nose itself prevents large particles (10 microns and larger) from entering, for they are stopped by inertia. The nose is lined with hair which has a ciliary action, sweeping foreign particles back towards the outside. Enzymes are present in the nasal secretions which have bactericidal activity. The secretions themselves wash the particles away. Any foreign materials reaching the lungs are exposed to protective action of the white cells (phagocytes). The skin acts as a barrier as do the protective fluids in the eyes, for the tears contain antimicrobial substances. The body fluids and tissues are normally protective. There are normal antibodies, white cells and other germ destroying cells throughout the body in case organisms get through the first lines of defense.

With all these barriers for protection, one wonders how and why organisms can cause infections. The germs in small droplets or dust particles or even as single cells may get into the eyes, ears. nose and throat or into wounds and extensive burns and even operation incisions or small breaks in the skin barrier. A few organisms may be taken care of by the normal body defenses. However, large or even small numbers of highly pathogenic germs, plus breaks in the body defenses such as occur in people already sick with another disease or recovering from operations, provide ample opportunity for infections to take place. It has been shown that a continuous exposure to very small numbers of organisms can eventually wear down body resistance to cause infections.

What has all this to do with AC and engineering and hospitals? The "bible" (ASH and VE Guide) states that air-conditioning is the simultaneous control of all *or at least the first three* of those factors controlling the physical and chemical

conditions of the atmosphere within any structure. These factors include: temperature, humidity, motion, distribution, dust, bacteria, odors and toxic gases, etc. When one considers the comfort provided a patient by temperature and humidity control, comfort which speeds up his recovery time in the hospital, then AC is certainly warranted. The more comfortable working conditions for the physician and surgeon while carrying out delicate operations should also be considered. For these reasons alone, no one should seriously consider hospital construction, in the Gulf Coast area at least, without AC.

The people involved in hospital AC have different ideas on what is necessary and what should be provided. The engineers sell temperature and humidity control, plus distribution. The physician, surgeon and health people (and patient too) want and need temperature, humidity and infection (purity) control. But what does the hospital administrator purchase? He wants temperature and humidity control at the cheapest possible rate —and apparently in most instances that's *all* he is interested in! He would accept 100% recirculation of air under certain circumstances and many times gets it. This is potentially very dangerous, since noxious gases as well as harmful organisms, allergens and dust can accumulate and make an open sewer of such an AC system. This problem could be met and solved if all of the interested groups could get together and pool their knowledge and ideas; for each while limited by the training and background of his own field, could aid in development of plans for complete, overall needs and requirements of hospital AC.

The field of air disinfection is still in its infancy. The methods are, for the most part, in the experimental stage. Some of them are of doubtful or questionable value in the control of airborne organisms and diseases. Much of the reported work has been carried out in army and navy barracks, some of it in schools and hospitals, but very little has appeared on air disinfection in conjunction with air-conditioning systems.

Ultra-violet radiation in the range of 2,537 Angstrom units has been under investigation for a number of years as a means of controlling diseases transmitted by air. Experimental studies and room trials indicate that 1–7 ergs/cm.^{2}/sec. of radiant energy should be attained for practical applications. The studies have revealed that bacterial populations may be reduced up to

90% while infection rate may decrease approximately 25%. Ultra-violet has been used extensively as a "curtain" between infectious and non-infectious areas in hospitals and laboratory buildings. Ultra-violet radiation can be dangerous to humans, for extended exposure may cause eye and skin irritation. It is relatively slow in bactericidal activity, but its action is additive. Recent investigations suggest that, under certain conditions of exposure to ordinary light, its action on organisms may be reversed or neutralized. It is of great practical importance that organisms may be shielded from UV bactericidal activity by being coated with, or by hiding behind, dust. This has been explained on the grounds that UV is absorbed by and will not penetrate well, through dust.

Extensive investigations with chemicals such as lactic acid, hypochlorite, essential oils and numerous others as bactericidal aerosols have yielded some practical results. The glycols have probably shown the greatest effectivity. Of the glycols studied, the propylene derivative is the most effective, although triethylene glycol has also shown promising results. Propylene glycol is active when dispersed as an aerosol in concentrations of 1–3 ppm. Pharmacological studies reveal that it is quite non-toxic even when the air is saturated with it. It has been shown to reduce bacterial air populations approximately 90% with concomitant infection rate drops of near 30%. The glycols do have some drawbacks. Continual use results many times in films of the material forming on furniture, walls and other surfaces. Continuous vaporization with accurate concentration control are necessary. The humidity has a great effect on the usefulness with concentrations above and below 50% RH detrimental to bactericidal action. In use with AC, it has been observed that vaporization of the glycol into air streams under 50° F. results in condensation of the chemical in the duct system. Again, as with UV, the glycols' bactericidal action is limited when the organisms are coated by dust particles. Apparently the glycols are not effective on surfaces or on dust protected organisms, but must attack microorganisms directly while in the air.

Forced draft ventilation systems may use air wash systems of one type or another. A spray of water will trap most of the dust particles but is not too efficient. One of the main difficulties with a system of this type to take out small particulate matter is that

the system is not drained and cleaned frequently. With continual use, large amounts of dust and dirt and microorganisms can accumulate in the water—in fact, under certain conditions these organisms can multiply. If the system is not cleaned, then the air passing through the "air-wash" can pick up organisms and the air becomes more contaminated rather than less. At best, this procedure is not very efficient for removing infective microorganisms.

There are numerous types of "filters" available for AC units and systems. The usefulness of many of them is apparently limited to keeping the coils of the refrigeration unit clean. To put it bluntly, most of them in use would do fine in stopping small birds, butterflies and golf balls! As for the organisms, 1,000 side by side could go through without touching any part of the filter. As the filter collects larger and larger amounts of dust, it may become more efficient as a mat of dust collects on the outside. In some instances, this mat of dust could act as a source of organisms to further contaminate the air stream. From our own observations, we could say that the filters are not cleaned or replaced often. We would suggest here that the replaceable filter unit would be much more desirable. One set of units, which we observed, did not have the filters changed until the load got too much for the machine and it cut off. Then and only then were the filters removed. In at least one medium-sized unit (5 ton) no filter was present at all. This type of servicing may not be universal but it may be fairly typical. It might be a suggestion for AC dealers and suppliers to sell a maintenance and servicing policy with the units to prevent the public, and the hospitals as well, from abusing or misusing the air-conditioning units. Such misuse could lead to obtaining poor cooling results (reduce efficiency) and thus reflect back upon the AC suppliers and industry itself. Some efficient filters are available for air-conditioning systems. Some of these are spun glass-wool filters and are marketed by several manufacturers. There are other types, which are available, also designed to stop fine particulate matter with high efficiency.

Recently great progress has been made in the field of electrostatic precipitation of particles from the air. In this procedure all of the air is passed through a highly charged electric field and the suspended particles are charged and precipitated—removing

the dust, allergens, bacteria, and any other material from the air. In actual use, small units may be placed in hospital wards when they could be connected with or separate from the AC equipment. These small units work at a level of approximately 12,000 volts and do not produce excessive or offensive amounts of ozone from their electrical discharge. Larger units are obtainable up to approximately 75,000 volts for use in large installations when such a unit might be placed in the air intake line so that all air entering a hospital could be treated to prevent outside contamination. This procedure is valuable in every day treatment of air and could be extremely valuable in time of war when biological and radiological warfare might be potential hazards. At present such an installation might be considered very expensive but with proper maintenance these units are very practical since tests have shown such precipitators to have greater than 98% efficiency in removing bacteria when the particles were in the range of 1 micron in diameter. These units may be practical also in treating the air being expelled from highly contagious laboratories, wards, or complete buildings. Such a procedure would prevent the air from carrying large numbers of pathogenic organisms from, for instance, a tuberculosis hospital to other hospital buildings or to the near-by general public. Electronic precipitators on the intake or exhaust air would not protect individual sections in the hospital unless all return air was passed through the precipitation units. Again it would be stressed that although the initial cost is high and the efficiency is very high, the units work only as well as the maintenance and the servicing personnel.

One of the fundamental air-borne infection control measures is neglected by many hospitals. This measure is dust control. It is not unique to air conditioned systems, but it is just as important as in any system especially the forced draft ventilation systems. We have already indicated the importance of dust as a carrier for and a protector of microorganisms. One of the first steps in dust control would be the collection and burning of all the sweeping-brooms found in a hospital. Apparently all of the janitorial personnel have instructions that when there is nothing else to do they should grab a broom and sweep just to keep busy or to look busy. The amount of dust and organisms sent up into the air as an aerosol by sweeping is tremendous. Literally mil-

lions of organisms may be suspended in the air and repeatedly resuspended each time a room is swept. These organisms may be breathed in or fall on wounds and burns or get on the "sterile" instruments and bandages. Some of the large particles settle immediately but the smaller ones (less than 10 microns) may stay suspended and circulate in the ventilating system. Some of the dust eventually collects on the filters of the AC units. This dust in the form of a mat eventually cuts down on the efficiency of the AC unit. Bacterial counts on the dust from AC filters show that it may contain several hundred thousand per ounce. Use of sweeping compounds cuts down the dust aerosols considerably as does wet mopping. During the past war oil compounds were found to be efficient for treating floors and blankets in hospitals. The oil mixtures contain germicides which may remain active for prolonged periods. British investigations suggest as a possible solution to the dust problem that vacuum lines be run throughout the hospital for vacuum cleaning to do away with sweeping or mopping of any type. The dust control should not be limited to the floor alone for the dust may settle on any surface. Experience has shown that other important sources of dust, lint and microorganisms in the air are from bedmaking or bandage changing.

An advantage in the control of air-borne infection through AC systems is the fact that in conditioning the air by removal of moisture, a number of organisms are killed. Many bacteria cannot withstand desiccation (drying) at ordinary room temperatures. It happens that the 50% RH sought for physiological comfort is also in the range for the most efficient glycol bactericidal action.

A number of factors affecting the bacterial population of the air have been considered as well as procedures for controlling air-borne organisms under hospital conditions. Each procedure has certain advantages and disadvantages. Each would work best for certain types of installation, needs and conditions. No one method should be used without considering the requirements of the particular problem at hand. The effect of humidity on each method should be considered. Of great importance is the role of dust control itself on the effectiveness of any procedure in controlling air-borne microorganisms, for inadequate dust control may defeat the purpose of any procedure designed to

eliminate these organisms and air-borne infection. In many instances a combination of the procedures might have great value. Electrostatic precipitation of the intake air plus ultra violet or glycol treatment of particularly vulnerable areas such as contagious wards, surgery units and nurseries are examples.

Laboratory workers have developed test methods and procedures for evaluating potential air-borne infection hazards and the control of air-borne infection hazards and the control of air organisms through UV radiation and glycols. Air samples may be obtained to determine the relative increase or decrease in the bacterial population resulting from any given procedure. Air sampling for microorganisms consists essentially of collecting the organisms from a measured volume of air either on the surface of a *solid* nutrient agar plate or in a *liquid* such as water or a nutrient broth preparation.

Many air sampling procedures and variations of procedures have been used with relative success. An early procedure was to expose agar plates for varying times in rooms to measure the settling rate of organisms from the air. This would give a picture of the number of larger particles in the air. The number could be determined after the plates were incubated at body temperature to allow the organisms to grow into individual colonies. An air centrifuge was developed which could sample air in test areas and rooms but had the disadvantage of sampling only the relatively large particles with little information being obtained concerning the very small particles or single cells which might be suspended in air and which would be most important in spread of disease.

Another type of sampler is the sieve-sampler. With this device, the air is drawn onto a nutrient agar surface through a metal disc having many pin-point holes. Knowing the amount of air sampled per unit time it is quite easy to calculate the number of organisms in the air by the number of bacterial colonies developing on the plate after incubation. This procedure has many advantages of size, cost and ease of handling and sampling.

Another agar surface sampling procedure utilizes electrostatic precipitation of the particulate matter onto the agar surface. This device is very efficient and portable but its usefulness is limited by the high unit cost.

A most practical modification of the agar plate sampling procedure is the slit-sampler. This apparatus has the great advantage of being able to sample on the same plate for an extended period. A mechanism turns the plate slowly so that the air coming through a small slit strikes only a small segment of the plate at one time. Thus any minute change in the bacterial flora from minute to minute can be measured. The rate of rotation can be varied depending upon the type of air being sampled or requirements of the particular studies being carried on.

Air sampling by liquid impingers has been used successfully by many investigators. Not only can one sample for organisms in the air but dust counts may be determined as well. The general procedure is as follows: A measured amount of air is bubbled through a known amount of liquid and then the liquid is sampled for the number of organisms by plating out on nutrient agar for the number of colonies developing. By this means the number of organisms in the original air sample may be determined. Dust counts may be made by observing the liquid microscopically to count the number of dust particles. The bacterial count from the liquid impingers is usually higher than the agar surface impinging method since the bubbling breaks up many clumps of organisms which may develop only as a single colony when sampled on the agar plate.

Various other sampling devices have been used. Less frequently the organisms have been collected in a cotton-filled tube by drawing air through and then suspending the cotton in water to remove the organisms for plating to enumerate them. Other tubes have been filled with sand as the collecting medium. Recently soluble gelatin foams have replaced the cotton or sand collectors in some investigations. In the investigative phase is the cellulose—paper filter having micropores, filters which can later be impregnated with medium for growing the organism right on the filter disc where they become impinged.

Since it may be difficult to evaluate the results of the various control measures if the original bacterial count of the air is very low, it has been found practical to use an "indicator" organism in certain studies. A non-pathogenic or non-dangerous organism may be sprayed into the air and samples taken at various points of the system at different times to determine the

efficiency of the particular test procedure to remove or destroy the organisms. An easily identified organism is the one of choice, one which has characteristics of survival similar to the usual air-borne infectious organisms. Such a procedure allows one to follow the death or removal of organisms more readily and makes comparison of procedures more valid since statistical evaluation may be applied more readily.

A search of the literature reveals that there has been no recent or comprehensive study of the role of air conditioning in the spread or control of air-borne organisms causing infectious diseases. Very little investigative work has been reported on the study of various air-borne infectious control procedures with AC. The need for air-conditioning has been indicated for the comfort of both patient and physician. It is important that the sick and injured get "clean" or purified air. Air that is free of potentially dangerous organisms is especially important for those patients with extensive wounds and burns since these may act as portals of entry for infective organisms. The opportunity for studying AC alone and with air-borne infection control measures presents itself now under conditions for a good experimental study. At the present time in our industrialized areas large numbers of industrial (chemical and other) burns occur. This is especially true in the Gulf Coast area.

With the advent of war, the number of extensive wound and burn cases would increase tremendously especially with possible atomic warfare. It has been revealed that many of the casualties of atomic bombing would be actual thermal burn cases.

In the past, burns were treated primarily with pressure bandages. In large scale burn groups each year many tons of bandages and hours of labor go into changing these dressings and applying new ones besides the cost of materials and man hours.

In the past few years another type of treatment has been made practical for individual burn cases and large scale burn casualties. This is the "open-air" method of treatment which has been made possible only through maintenance of proper nutrition, and the use of plasma and antibiotics. Under this treatment procedure many types of burn patients may be maintained without any pressure bandages at all. In this manner the burn surfaces dry, heal more rapidly, grafting of skin where needed

may be carried out earlier, less scarring and disfiguring occurs, and the patients are able to recover and leave the hospital sooner.

Of the hospitals interested in this type of treatment, one of the main ones is the Special Surgical Unit of the John Sealy Hospital, The University of Texas, Galveston under the direction of Doctor Truman Blocker. Here many burn cases are treated each year. Here is presented a fertile field for AC studies since almost identical ward conditions are available with and without air-conditioning. These patients come into the hospital with many organisms on their body surfaces. These organisms are potentially dangerous to the patient as well as the other patients since they can get into the air through the many avenues mentioned previously and could pass from one patient to another to cause infections. Preliminary surveys of such wards show large numbers of organisms in the air. In such wards a well controlled survey could be made of the factors affecting survival and spread of organisms in hospital air. A comparison of the bacterial flora of the AC and non-air conditioned wards could be readily conducted. Of greatest value with this type of installation where both types of wards would be available would be an evaluation of various types of air-borne infection control measures in conjunction with and without air conditioning equipment. Especially important is the determination of the effect of air-conditioning factors on the germicidal effect of the air-borne infection control measures.

It is suggested that the individual control measures be tested alone and then combinations of the various procedures may be evaluated when operated concurrently. Since there might possibly be some residual effect of some of these procedures, they might best be tested in the following order: (a) filtration, (b) ultra-violet radiation (in room and in AC system as well), (c) electrostatic precipitation, (d) glycols, (e) dust control, and then (f) combinations of the above control procedures.

The effects of the various control procedures could be followed directly by air sampling for changes in the bacterial flora and sampling of the dust throughout the wards for bacterial population counts. The results might also be determined indirectly by following the recovery of the patients in the different wards under test. This would include comparing the incidence of infection, rate of skin healing, amount of scarring,

other complications and the overall rate of recovery and length of stay in the hospital.

Above has been outlined a practical application study of air conditioning (AC) under hospital conditions to determine its possible role in the spread or control of infectious organisms and to evaluate air-borne infection control measures in conjunction with AC to determine the efficacy of such procedures with AC equipment and under such factors as temperature and humidity levels of AC systems. In such a study under "field conditions" many of the variables and factors cannot be controlled or changed at will because the hospital routine must go on. It is suggested therefore that besides the hospital ward studies an experimental room study should be developed whereby temperature, humidity, rate of air flow and air change, filters, UV, electronic precipitators, glycols, and dust control may be varied at will using indicator organisms where necessary to give an exact determination of the effect of the variable factors and test procedures on the bacterial population and flora of the air under conditions produced by AC methods.

It is felt that hospital AC presents a definite problem which bears a thorough investigation. The problem is present not only for new installations in completely air conditioned buildings but for package units for wards, clinics, and surgical amphitheaters and suites or even for small fractional-ton units for individual rooms. Many questions arise as to proper design and location of the intake and exhaust. There is always a question of whether there should be any recirculation of air in certain sections of the hospital and if so what percentage of air should be recirculated. Such a problem always arises with surgical suites and nurseries. Proper maintenance of equipment is a major problem. The use of proper filters is important as is the routine cleaning or replacement of filters as well as the cleaning of the equipment itself. The proper education of the users in the correct use of AC is paramount. This includes the administrator, staff and maintenance personnel as well since all play an important role in proper use or misuse of the equipment.

Various other problems arise in conjunction with AC, some of which are forgotten or ignored. One of the simplest to control and the easiest to forget is the correct cleaning of the hospital to alleviate dust. The control of dust makes the other measures

much easier to operate successfully. Among the other problems is the suppression of noise of machinery and the forced draft ventilation noises for hospital quietness. The vibrations from the heavy equipment may seem negligible to the average person but laboratories using sensitive equipment and microscopy find the slightest vibration to interfere with their work. Another of the hazards arising from forced draft ventilation and AC is the dispersal of molds through the system. In bacteriological laboratories of hospitals this causes much trouble in attempting pure culture work in diagnosis of disease. The molds may also act as allergens for some of the patients and staff.

The problems involved for proper installation, use and maintenance of hospital AC are the problems of not only the engineer and supplier of equipment but the hospital administrator, staff doctors, health personnel and the maintenance crew. As indicated above, the problems can best be worked out by the combined know-how of each interested party as a group. Since the American Society of Heating and Ventilating Engineers has a big stake in the best possible utilization of AC equipment it is to be recommended that they sponsor this type of basic research and field investigation. In sponsoring the research they could also supply their engineering talents in consultation to assure correct engineering principles in the experimental studies and to translate the findings of experimental data to installation of both large and small AC equipment as well as to correcting or modifying present installations to insure proper control of air-borne infecting agents in hospitals.

THE EFFECT OF VARIOUS TUMOR GROWTHS ON THE WATER CONTENT OF THE LIVER IN MICE AND CHICK EMBRYOS*

JOHN C. KNOX, JR., HILDA F. ROSENE, AND ALFRED TAYLOR

In 1935 Okuneff and Nasarbekowa demonstrated that there was an increased water content of neoplasms. Subsequently, confirmations were reported by Stern and Willheim (1943), and by Greenstein (1947). Actually, an increased water content of neoplasms would be expected since a high water content is characteristic of young growing tissues, especially embryonic tissues. Experiments have been reported which indicated that the tissues of tumor-bearing hosts contain varying amounts but definitely higher water content than analogous tissues in normal animals (Schlottman and Rubenow, 1932 and Greenstein, 1947). The tissues of the cancerous host are not growing yet they reflect indications of the effects of the cancer (Greenstein, 1947), for example in the liver it has been observed that there are changes in its hemoglobin concentration, sinusoidal dilatation, and in water content.

Schlottman and Rubenow (1932) reported that there was an increase in the per cent of water in the liver of rats which had been injected subcutaneously with Flexner carcinoma and Jensen sarcoma. McEwen and Haven (1941) inoculated rat carcinosarcoma 256 directly into the liver of rats of the Wistar strain and found an increase above normal of the per cent of water in the liver. They found also that there was an increase in the water content of the liver where the tumor growth was introduced subcutaneously. The results of McEwen and Haven (1941) show that the tumor reached a certain size (10 gms.) before a change in the per cent of water in the liver, significantly higher than that of the normal rats, occurred, and that after this size was attained, further increase in size of the tumor produced no similar increase in the per cent of water. They concluded that this increase in the water content of the liver was not the result of a decrease in liver dry residue. Annau and co-

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workers (1951b) suggest that the increase of water content found by McEwen and Haven may be related to some structural changes in the cytoplasmic pattern of the cell.

More recently, Yeakel (1948) reported on changes in liver weight as compared to body weight and body length in Wistar albino rats bearing subcutaneously induced or transplanted tumors. She compared the weights of mammary gland tumors with the weights of the hosts' whole liver and found no correlation, except that when the size of the growth exceeded 30 gms., enlargement of the organ occurred. She found also that the weight of the liver relative to the weight of the body was significantly increased when the body length of the rat was over 175 mm., but further growth of the tumor showed no correlation with a proportional increase in liver size. There was a more pronounced effect in the female rats than in the males. In conclusion, Yeakel (1948) suggested that liver enlargement is connected with protein anabolism during growth of the tumor.

Autopsies carried out by Annau and coworkers (1951a) on rats and mice bearing spontaneous or grafted tumors demonstrated a relationship between the weight of the tumor and that of the liver/body ratio (L/B). Their experiments included both sexes of the animals which were of various weights, and which supported one of the following types of tumors: reticuloendothelioma of the rat, fibrosarcoma of the albino mouse, Meyer tumor (a mammary tumor of Brazilian origin) of the albino mouse, spontaneous, and transplanted mammary carcinoma of the C_3H strain mouse. The authors point out that their findings are in agreement with those published by Yeakel (1948), and that in general it seems that liver enlargement of the host animal is detectable even when the size of the growth is relatively small, but it is most pronounced with larger tumors. The authors maintain that the degree of enlargement of the liver is apparently dependent upon the ability of the liver to respond to stimuli released by the tumor. They also believe that a variety of factors may be involved in this phenomenon, that the results of McEwen and Haven (1941) suggest the possibility of systematic changes in the cytoplasm, and that Yeakel's (1948) conclusion (that this increase is induced by hypertrophy leading to an increased protein anabolism) remains to be proved.

Based upon their histological investigations, Annau *et al.* (1951a) concluded that the increased weight of the liver may be explained in part by a mitotic activity induced by the presence of the tumor. Their controls showed no trace of mitotic figures. In a later report (Annau *et al.*, 1951b) they state that the obviously altered mitochondrial pattern and mitotic activity of the liver might be explained as the result of extreme hyperactivity if the concept of Yeakel and Tobias (1951), that the liver of animals bearing tumors present a picture of "work-hypertrophy," is correct.

During the course of an investigation by Kynette and associates (1945) on the effects of yolk sac tumor growth on the chick embryo, they noted varying degrees of liver disturbance and that this organ appeared to be more affected than any other visceral organ. They noted also that when there was a large tumor present, the size of the liver increased considerably. In addition to this change in liver size, they found other gross changes varying with the severity of the growth. From histological sections of livers with severe and less severe tumor growths, corresponding variations of structural abnormalities within the liver were revealed, thus indicating a definite relationship between the size of the tumor growth and its effect upon the liver. The authors concluded that the increased liver size in the chick embryo might be due to the increased activity of the blood-forming function of this organ.

Skavinski and Stein (1951) reported an increase in the rate of growth of the liver affected by the growth of tumor implants on chick embryos. They interpreted this change as a readjustment on the part of the liver in an effort to meet the metabolic requirements of the embryo.

Klatt and Taylor (1951) recently observed a marked increase in liver size of tumor-bearing mice and chick embryos in all experimental groups bearing six different types of tumors. They concluded that the presence of a tumor in an animal is associated with changes in the liver which result in an increase in weight without a corresponding increase in active liver tissue.

It is evident from the work which has been done on rats that there is no general agreement concerning the conclusions of whether the rate of growth of the tumor is in proportion to the increased whole weight of the liver, or to the increased water

content of the liver, or both. Up to the present as far as the author is aware, no investigation has been reported on growth of the tumor and weight of the liver in relation to water content in the liver of mice and of chick embryos.

Materials and Procedure

The materials used were the common house mouse *Mus musculus*, male and female of the DBA strain, and embryonated eggs. It was felt not necessary, for this particular study, to include body weight and body length of the animals. One hundred thirty-six mice and 142 embryos were employed, with the experimental group of mice bearing 6 types of tumors and the experimental series of chick embryos supporting 2 types of transplanted tumor growths in their yolk sacs. Taylor's technique (1948) was used to inject the tumors.

Controls for the mouse series of experiments consisted of stock breeder mice of the same sex, approximately of the same age, and of the same strain as those used in the experimental group. They were maintained on the same diet of Purina laboratory chow and were housed under the same environmental conditions. The controls had not previously been injected with tumor tissue, that is, they were not experimental animals which failed to take an injection of the tumor suspension. Up to the time of being sacrificed, the control mice showed no external appearances of a tumorous growth.

The controls for the experimental group of embryonated eggs were normal incubating eggs and were incubated with the experimental series at 37.2° C., thus excluding variations in treatment of the eggs or in the incubation temperature. Four different periods of incubation were used in order to provide a broader basis of study for this investigation.

Since all 278 dissections of both mice and chick embryos were done by one individual, it was possible to maintain uniformity in the procedure. It was of the utmost importance that the dissection not only be fast but also consistent and accurate. Once the peritoneum was opened and the abdominal cavity exposed, evaporation from the liver takes place within a matter of minutes.

The mice were lightly etherized and then decapitated. Particular care was taken to decapitate the animal just before

death so that a maximum flow of blood could be obtained, thus ensuring a standardized bleeding procedure. After allowing the animals to bleed thoroughly, an incision was made in the abdomen and its cavity exposed. The peritoneum was then cut, the cardiac cavity was opened to snip the vessels entering and leaving the heart, and supporting tissues and nerves were excised from the liver to provide its rapid extirpation, thus reducing possible evaporation. All non-hepatic tissue, stagnant blood and exudate were quickly removed from the liver before placing in the drying vessel. This operation usually took about one minute, that is, from the time the abdominal cavity was exposed until the organ was placed in the weighing vessel. The tumor growths were dissected and weighed on a pan balance. Quite often, the tumors of mice are necrotic. Necroses of a severe nature, that is, open and draining, were discarded since this would be a source of variation in the results.

The vessel containing the wet liver was weighed to the nearest milligram on a chainomatic balance and placed in a drying oven to dehydrate between 55 and 60° C. for 24 hours, after which the container was removed and allowed to cool for a few minutes before weighing to the nearest milligram. Subsequently, to insure a constant weight, four successive weighings were made an hour apart. Between each weighing, the vessels were replaced in an oven for one hour of further drying.

As a rule, the chick embryos were dissected as soon as they were taken from the egg; however in a few instances, they were chilled and kept for as long as four hours. In this case, control embryos were treated in a similar manner. Unpublished data by Taylor indicates that chilling has no effect on the embryo or on the results of the experiments. Usually four eggs were opened in succession and the embryos set aside to allow maximum bleeding before dissection. The dissection technique was essentially the same as that used in the mouse series. The small size of the chick embryo liver necessitates special care. The size of the tumor growths cultivated in the yolk sacs were usually estimated. Upon comparison of these estimates with determined weights of the same tumor, negligible variations were found.

Greenstein (1947) has pointed out that the primary source of variations in the data of different investigators arises from the material that is chosen for their particular study. Hence, by

using mice of the same strain, maintained upon the same diet and by using eggs from the same flock of chickens and incubated together, reliable results would be obtained.

Results

The data obtained on the effect of tumor growth on the percentage of water content in the liver of mice are summarized in Table 1. The comparable results obtained using tumor-bearing embryonated eggs are given in Table 2. The data in Table 1 are grouped according to type of tumor, sex of mouse and arranged in each column in order of increasing size of tumor growth. The data in Table 2 are grouped according to the period of incubation and the type of tumor employed and also arranged in order of increasing size of the tumor.

1. *Spontaneous Tumor.* The data in Table 1 from the group of mice bearing a naturally occurring tumor show that in every instance of increasing tumor size, there was also an increase in the average wet weight of the liver. There is a similar relationship between the growth of the tumor and the per cent of water content in the liver. In the presence of a 0.6 gm. tumor, there was an increase of 8 per cent in water content above that of the controls, with the controls equal to 100. With a 9.0 gm. tumor, the increase amounted to 69 per cent. The variations found in the average weight of the dry liver and the average weight of the whole liver on a percentage basis will be considered later. With this particular growth, there was only one instance of a decrease in the per cent of active liver tissue below that of the controls. Only female mice were used in this series of experiments since males rarely develop a spontaneous tumor. Figure I shows the proportionate effect of this tumor growth on the weight of the liver and on its increased water content.

2. *DBA Sarcoma 131.* With this type of tumor growth both male and female mice were employed as host animals. In the female group of mice bearing the transplanted spindle-cell sarcoma, there was one variation in the series and this was found in the average dry weight of the liver where those individuals bearing a 1.6 gm. tumor had a lower dry weight of the liver than the controls had (Table 1). There was, however, an increased water content correlated with the increased weight of

TABLE 1

The Effect of Transplanted and Spontaneous Tumor Growths on the Size and the Water Content of Liver in Mice

	No. and Sex	Av. Tumor Weight (gms.)	Av. Wet Liver Weight (gms.)	Av. Wet Liver Wt. Where C = 100	Av. Dry Liver Weight (gms.)	Av. Dry Liver Wt. Where C = 100	$\frac{\text{Dry}}{\text{Wet}} = \%$	$\frac{\text{Dry}}{\text{Wet}} = \%$ Where C = 100
			Spontaneous Naturally Occurring Tumor					
Control	25 ♀		1.33±0.17		0.42±0.09		31.6	
Exptl.	5 ♀	0.6±0.5	1.44±0.50	108.3	0.49±0.20	117.6	34.0	109.2
	9 ♀	2.2±1.2	1.48±0.80	111.3	0.47±0.27	111.9	31.8	100.9
	6 ♀	4.3±2.1	1.73±0.56	130.1	0.46±0.18	109.5	26.6	84.6
	4 ♀	9.0±1.0	2.25±0.33	169.2	0.72±0.16	171.4	32.0	101.2
			Spindle-Cell Sarcoma 131 (Transplanted)					
Control	20 ♀		1.15±0.54		0.37±0.18		32.2	
Exptl.	5 ♀	1.6±1.0	1.21±0.17	105.2	0.35±0.02	94.5	28.9	89.8
	7 ♀	2.3±1.0	1.35±0.40	117.4	0.38±0.09	102.7	28.1	87.5
	5 ♀	2.9±1.9	1.45±0.23	126.0	0.42±0.08	113.5	28.9	90.1
			Spindle-Cell Sarcoma 131 (Transplanted)					
Control	20 ♂		1.22±0.48		0.38±0.20		31.1	
Exptl.	5 ♂	1.4±0.4	1.08±0.30	88.5	0.32±0.08	84.2	29.6	95.1
	5 ♂	2.0±0.4	1.17±0.12	96.9	0.34±0.04	89.5	29.1	92.4
	5 ♂	3.4±0.7	1.22±0.48	100.2	0.34±0.11	89.5	27.9	89.5
	5 ♂	4.5±1.0	1.34±1.14	109.8	0.32±0.04	84.2	23.9	76.5
			DBA Mammary Carcinoma (Transplanted)					
Exptl.	3 ♂	1.7±2.3	1.30±0.20	106.6	0.41±0.02	107.9	31.5	101.2
	3 ♂	4.1±1.0	1.41±0.20	115.6	0.43±0.05	113.2	30.5	97.9
			Lymphosarcoma (Transplanted)					
Exptl.	4 ♂	2.5±0.0	1.81±0.17	148.4	0.45±0.04	118.4	25.0	79.8

the tumor growth, above that of the control series. The water content increased from 5.0 to 26 per cent in association with increasing tumor size. In every instance, there was a decreased percentage of active liver material. In the male mice injected with this type of transplant, the effect tended to be comparatively less which seems to indicate a possible sex difference of this type of growth. The average wet weight of the liver and the per cent of water content present in the organ did not increase until a 3.4 gm. tumor growth was attained and then with further increase in size of the tumor, there was found a similar increasing correlation between tumor size, liver weight and water content. However, the per cent of active liver tissue was still found to be below that of the control value.

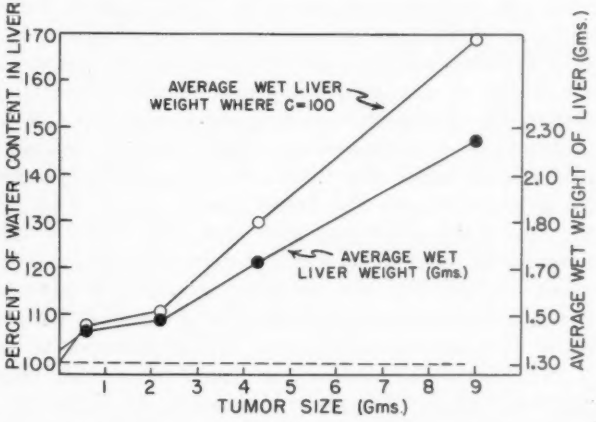

FIG. I. Graphical illustration of the effects of a spontaneous tumor on DBA female mice showing the relationship of the rate of tumor growth with the weight increase of the whole liver and the amount of water content present in the liver above the control value of 100 per cent. Data is based on Table 1.

3. *Transplanted DBA Mammary Carcinoma and DBA Lymphosarcoma.* In both of these groups, there was an increase in the weight of the liver and the water content of the liver as shown by Table 1. In the animals bearing a 4.1 gm. mammary carcinoma, the increase in water content was 15 per cent above that of the controls. With the lymphosarcoma of approximately

2.5 gms., there was a 48 per cent increase. Comparing the latter tumor growth with the various tumor growths in the preceding experiments, we find that the lymphosarcoma affected the host animal to a more marked degree. The controls for this series were the same as those used for the sarcoma 131 male group of experiments.

4. *C_3H Mammary Carcinoma.* The data in Table 2 demonstrates that the effect of the growth of transplanted mouse and rat tumor tissue in the yolk sac of chick embryos on the liver followed the same trend as that found in the mouse series. The periods of incubation with the C_3H mammary carcinoma were 13, 14, and 16 days. With a tumor transplant incubated for 13 days and weighing 0.5 gms., the water content increase was only 3 per cent but with the largest tumor size of 2.2 gms., the increase was 131 per cent above that of the controls. In every instance, as in the mice, the per cent of active liver tissue was seen to be below that of the controls. From the growth of a tumor in the 14 day incubated chick embryo, the data show similar results. The percentage increase of water content in the liver of the 14 day incubated egg ranged from 18 to 88 per cent with increasing tumor sizes from 0.5 to 1.6 gms. The per cent of active liver tissue was also below that of the controls. With tumor sizes of 0.9 to 2.8 gms. associated with a 16 day incubation period, the increase in water content ranged from 27 to 79 per cent. As found in the preceding experiments with mice, there was a decrease in the per cent of liver material. The curves in Figure II A show the effect of the 13 day incubation period of the C_3H mammary carcinoma as a typical trend found in other groups with different periods of incubation (Cf. Table 2).

5. *Transplanted Rat Sarcoma.* In this series of experiments with a single incubation period of 18 days, although there was an increase in weight of the liver with the increasing tumor size, this increase was never above that found in the control group (Table 2). Similarly, there was no increase in the water content in the liver with increasing tumor size. With a 1.0 gm. tumor the weight of the liver was decreased by 0.078 gms. below that of the controls, and was 17 per cent below the value for controls. However, with the largest size tumor of 2.7 gms. there was a decrease in the per cent value to only 6.5 per cent showing an actual increase in water content as the tumor was grow-

TABLE 2

The Effect of Yolk Sac Cultivation of Tumor Growths on the Size and the Water Content of Liver in Chick Embryos

	No.	Av. Tumor Weight (gms.)	Av. Wet Liver Weight (gms.)	Av. Wet Liver Wt. Where C = 100	Av. Dry Liver Weight (gms.)	Av. Dry Liver Wt. Where C = 100	Dry / Wet = %	Dry / Wet = % Where C = 100
			C$_3$H Mammary Carcinoma		13 Day Period of Incubation			
Control	19		0.146±0.063		0.029±0.016		20.0	
Exptl.	19	0.5±0.4	0.150±0.055	102.7	0.026±0.008	89.7	17.3	87.4
	10	1.1±0.8	0.238±0.090	163.0	0.040±0.017	137.9	16.8	84.7
	9	2.2±0.9	0.338±0.062	231.5	0.058±0.008	200.0	17.2	86.2
			C$_3$H Mammary Carcinoma		14 Day Period of Incubation			
Control	17		0.153±0.032		0.030±0.007		19.6	
Exptl.	6	0.5±0.3	0.180±0.011	117.7	0.033±0.002	110.0	18.3	93.2
	12	1.0±0.2	0.226±0.052	147.7	0.039±0.010	130.0	17.3	87.8
	5	1.6±0.0	0.288±0.092	188.2	0.046±0.010	153.3	15.9	81.4
			C$_3$H Mammary Carcinoma		16 Day Period of Incubation			
Control	18		0.251±0.192		0.058±0.023		23.1	
Exptl.	10	0.9±0.5	0.319±0.117	127.1	0.068±0.022	117.2	21.3	92.1
	10	1.8±0.4	0.372±0.065	148.2	0.074±0.014	127.6	19.9	86.5
	7	2.8±0.5	0.450±0.114	179.3	0.082±0.062	141.4	18.2	78.8
			Rat Sarcoma		18 Day Period of Incubation			
Control	16		0.459±0.088		0.126±0.032		27.4	
Exptl.	9	1.0±0.8	0.381±0.117	83.0	0.093±0.024	73.8	24.4	88.9
	9	1.9±0.3	0.386±0.029	84.1	0.094±0.003	74.6	24.1	88.7
	6	2.7±0.4	0.429±0.138	93.5	0.096±0.026	76.2	22.4	81.5

ing but never reaching the value of 100 for the control group (Cf. Figure II B). The decrease in per cent of liver dry residue was still apparent, however.

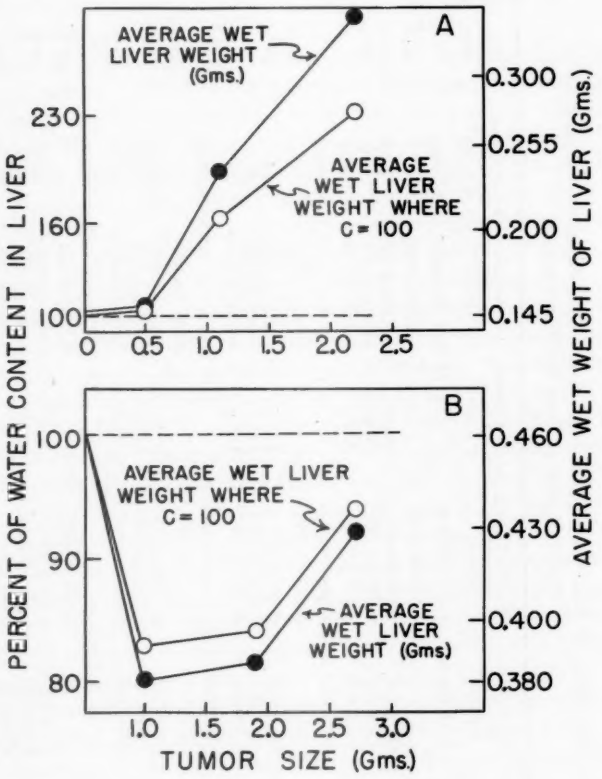

FIGURE II

A. Curves which show the rate of tumor growth of a C_3H mammary carcinoma in a 13-day incubated chick egg, plotted against the weight of the liver and its water content where the controls equal 100 per cent.

B. Curves showing an apparent decreased toxic effect of the transplanted 18-day rat sarcoma in the embryonated egg, on whole weight of the liver and the amount of water content present in the organ. Data in Figure II, A and B, are based on Table 2.

Discussion

The findings in this study on mice and chick embryos are in accord with Schlottman and Rubenow (1932) and McEwen and Haven (1941) who found an increase of the per cent of water in the non-tumorous liver when rats were used as tumor-bearing hosts. In addition, in mice and chick embryos, it was found that there was an increase in the fresh weight of the liver and per cent of its water content proportional to the growth of neoplastic tissues. The tumors of the mice included a naturally occurring spontaneous tumor, transplants of a spindle-cell sarcoma, a DBA mammary carcinoma, and a DBA lymphosarcoma, while Schlottman and Rubenow used rats bearing Flexner carcinoma and Jensen sarcoma, and McEwen and Haven used rats injected with a carcinosarcoma 256 tumor. The tumors in the embryonated chick eggs were transplants of a C_3H mammary carcinoma and a rat sarcoma. A proportional relationship between the increase in size of the tumorous growth, and increase in wet weight and water content of the liver is more evident from the data obtained with chick embryos.

By using the yolk sac method of cultivating tumor tissue in the embryonated egg, the data obtained have particular advantages over harboring the neoplastic tissue in a mouse environment and are found to be more consistent. Taylor (1950), has pointed out that:

> Perhaps the main advantage of the egg-grown tumor over the mouse transplant is the presence in the egg of fast growing non-tumor tissue along with the tumor. Such data as are available indicate embryo tissue to be biochemically and physiologically nearer to tumor tissue than any other non-tumor tissue. Hence, the reaction of the chick embryo serves as a rather delicate index of the specificity of any effects the yolk sac tumor may have.

Skavinski and Stein (1951) support Taylor's viewpoint. They state as follows:

> It was felt that the avian egg would provide a fully adequate system for testing the effect of the tumor on liver catalase, since it is free from the variable of a possible, but undetected, essential limiting substance in standard laboratory diets. Furthermore, the physiological adaptability of the embryo might allow it to adjust its metabolic processes to meet the demands imposed by both the normal developmental process and the growth of the tumor.

Tumors produced by the yolk sac cultivation method are contained in a relatively stable biological system. The method provides the study of a variety of tumors against a common background since the stroma of the neoplasm is furnished by the chick tissue.

The spontaneous tumor arising in stock breeder female mice presents a rather varied collection of connective tissue, epithelial cells and blood vessels besides the aggregate of tumor cells. This fact is apparently the cause of variations in the results obtained when using this type of tumor tissue in a systematic study of its effects. Other tumor growths employed in this investigation were transplanted hence, exhibiting a pure collection of tumor cells with few if any variations in results.

The data in Tables 1 and 2 show that the limit of increase in wet weight or in water content of the liver is not determined by the size of the tumor or continued growth of the tumor. In this respect, the data are contrary to those of McEwen and Haven (1941) and of Yeakel (1948) who found that the tumor growth reached a certain size before an alteration in the weight of the liver occurred yet further increase in size of the tumor failed to produce a similar increase in weight or in water content of the liver. McEwen and Haven found the values for the dry liver weight were not significantly higher than the control values. The data obtained in this study show that the control value of dry liver weight was exceeded by the dry weight of the liver in experimental animals except in the case of male mice with sarcoma 131 tumor and in the series of chick eggs inoculated with transplants of a rat sarcoma. Variations in data among investigators may be primarily the result of using different types of tumor growths in their studies.

An interesting point was brought out in the data by using the spindle-cell sarcoma 131. Since both female and male mice harbor this tumor equally well, both sexes were used as hosts. The livers of the female mice were affected to a more marked degree than were those of the male group. Yeakel (1948) reported that the influence of a tumor (transplanted fibrosarcoma or primary subcutaneous growths) on the weight of the host's liver was more evident in female rats than in males. Further work with mice bearing a spindle-cell sarcoma would be of value in that

inherited factors might be demonstrated as determining sex difference.

The data in Table 2 show that in embryonated eggs injected with transplanted rat sarcoma the tumor failed to produce an increase in the weight of the liver or the amount of water content present in the liver. This group proved to be the only case where the tumor growth showed this effect. This may be explained by an apparent decreased toxicity of the tumor tissue on the chick embryo, such as that mentioned by Taylor *et al.*, (1949) and later by Klatt and Taylor (1951).

Since the increase in whole weight of the liver appears to be firmly established, one is tempted to consider the cause of this increased size. This study corroborates the report by Klatt and Taylor (1951) that, in the presence of a tumorous growth, there is a decrease in the amount of active liver tissue, therefore eliminating the increase in liver size as being accounted for by an increase in liver material. From the data in the present investigation it is shown that the increase in size of the liver, even though there is a decrease in active liver tissue, is the result of retention of water within the organ.

Summary

An investigation was made of the effect of various tumor growths on the water content of the liver in mice and in chick embryos. A total of 136 DBA mice were used, the experimental groups of which bore subcutaneous transplants of a sarcoma, a mammary carcinoma, a lymphosarcoma, and a spontaneous mammary carcinoma. The tests included 142 chick embryos. The tumor-bearing eggs were inoculated in the yolk sac with a C_3H mammary carcinoma, and a transplanted rat sarcoma.

The evidence strongly suggests that a proportional relationship exists between the increase in size of the tumor growths and increase in wet weight and in per cent of water retained in the liver. Apparently, increase in size of the whole liver is accounted for by the presence of an increased amount of water in the organ and is not the result of an increase in amount of active liver material.

According to the data, female mice bearing a transplant of the spindle-cell sarcoma 131 were more affected by the influence

of the tumor than were males, thus demonstrating a sex difference of the tumor growth in this case.

The series of embryonated eggs bearing transplants of rat sarcoma in their yolk sac were found to be the only group in the experiments which failed to show an increase in fresh weight of the liver or in its per cent of water content.

The results of this study show that the effect of tumor growth on the weight and water content of the liver may be universal in its character since the general trend of the investigation persisted when various types of tumor tissues and various environmental backgrounds for the tumorous growth were used.

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CLOTHESPINS FOR LABORATORY GLASSWARE

LLOYD S. LOCKINGEN*

The use of non-standard Pyrex glassware in this laboratory necessitated the development of auxiliary equipment for such purposes as mounting growth flasks in constant temperature water baths, holding containers erect during autoclaving and in other operations. Most standard commercial clamps and fixtures are unsatisfactory because they will not accommodate the shapes

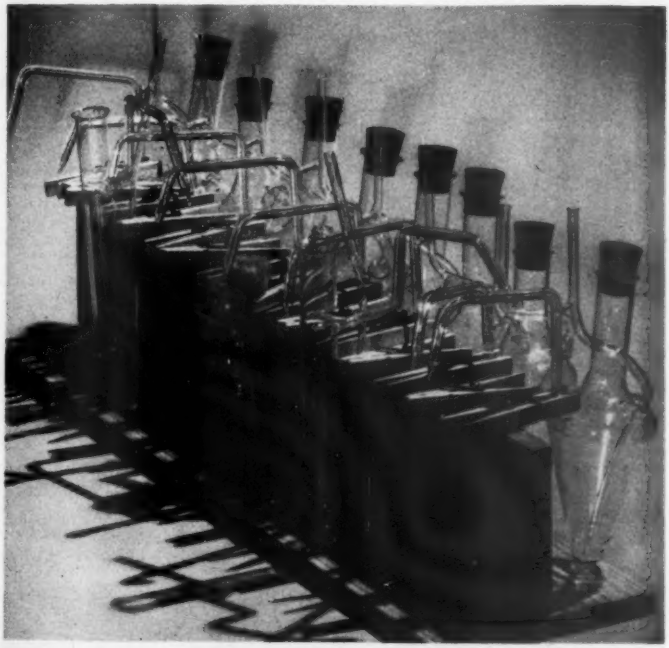

FIGURE I

of the glassware used and also because their use requires space beyond the capacity of such fixed equipment as the autoclave and the water bath.

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Several devices were developed but none have been as successful as those in which modified clothespins have been the holding agent. Four different adaptations of the common spring type clothespin and their uses are described as follows:

1. The clothespin is permanently attached to racks, tables, or other surfaces by means of a screw or nail installed through the hole in the spring. This method was used in the design of the autoclaving rack illustrated in figure I.

FIGURE II

2. The capacity of the standard clothespin is increased to accommodate larger diameters in the following manner: First, remove the springs from two pins; nail or glue two side pieces

together such that the jaw of the outer side piece protrudes past the end of the inner side piece; reassemble the two new side pieces with one spring. This method was used in the constant temperature water bath illustrated in figure II. The flasks are held by such enlarged pins mounted below the main rack by the method described above.

3. More rigid positioning is produced by removing the spring, nailing or gluing one side-piece to any suitable surface, and re-installing the spring and other side piece. This method provides a simple mount for wall charts, clip boards, manifold holders, rubber and plastic tubing, etc. The manifold illustrated in figure II is so mounted.

4. Temporary installations are made by slipping the clothespin spring over a finish nail or heavy wire fixed at the desired location. The temporary constant temperature water bath illustrated in figure III is one example of this method.

FIGURE III

In addition to the purposes described and illustrated, clothespins can be used in the laboratory for a number of other purposes. The methods described have provided unique and satisfactory solutions to otherwise difficult problems at a minimum expense.

TUMOR RESISTANCE PHENOMENA: IV. ACQUIRED RESISTANCE*

JOHN B. LOEFER AND ROY B. MEFFERD, JR.†

Extensive research over many years has served primarily to emphasize that acquired immunity responses to tumors are absent or weak (Woglam, 1929; Lumsden, 1931; Hadfield and Garrod, 1947, Chap. 6), and may be of a fundamentally different nature from clasical immunity responses to antigenic stimuli. That some degree of acquired immunity is present seems beyond dispute and it is possible that the development of more sensitive serological tests may yet reveal that any tumor elicits some type of antigenic response.

In our studies upon tumor resistance, it was essential that a clear-cut delineation be established between acquired and natural resistance.

Experimental

The fibrosarcoma was the same as that used in previous studies with Sherman-strain rats (Loefer and Gilles, 1951). Single grafts were made subcutaneously in the area of the right axilla, using the trocar method as previously described (Loefer, Mefferd and Nettleton, 1952).

Each experimental group actually served as its own control, since all animals within it were known to be susceptible to tumor implantation from a previous challenge, and in the absence of acquired immunity, should have implanted tumors in all cases. The groups labelled control in tables 1 and 2 are not controls of tumor takes, but actually of experimental procedures, since a certain proportion of rats exhibit natural resistance to tumor implantation under a given set of conditions.

Experiment 1 consisted of 3 series. In series 1, 9 rats, each bearing a tumor from a graft made 14 days previously, were given a second challenge with a similar-aged tumor in the same manner as the previous inoculum. A control group was grafted

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at the same time. In series 2, 23 tumor-bearing animals were given another graft, along with controls. In series 3, in which younger animals were employed, 55 tumor-bearing rats and 48 normal rats were challenged. The results indicate 63% takes in the tumor-bearing animals, whereas one might have expected these susceptible animals to have exhibited 100% implantation, had there been no acquired immunity. The results indicated, however, that a significant number of rats in this group were immune to the second tumor challenge.

TABLE 1

Incidence of a transplanted rat fibrosarcoma in tumor-bearing animals (A single stock tumor was used for grafting each series)

Series Number	Age of Rats–Weeks	Control Rats without Tumors: Rats with New Tumors	Total Rats	Percent Takes	Rats Bearing 14-Day Tumors: Rats with New Tumors	Total Rats	Percent Takes
1	7–8	13	21	62	6	9	67*
2	6–8	13	31	42	7	23	30*
3	3–5	30	48	62	42	55	76*
Totals and Averages		56	100	56	55	87	63*

*Expected incidence in absence of acquired immunity is 100.

In Experiment 2, 77 rats, 2–3 weeks old, were challenged with tumor grafts; tumors implanted 50 (65%). After 19 days, the tumors were excised, and 12 days later a second graft challenge was made. Fifteen (30%) of the animals were now resistant. As in Experiment 1, had there been no acquired immunity, one would have expected 100% takes rather than the 55% observed.

TABLE 2

Fibrosarcoma implants in rats from which tumors had been removed

	Age of Rats–Week	No. of Rats	No. with Tumors	Percent Takes
Controls—no previous tumor..	7–8	31	21	68
Susceptible test group*.....	7–8	50	35	70†

*Grafts had been made on these animals when 3 weeks old and the tumors excised 19 days later. The second series of grafts was made 12 days later.
†Expected incidence in absence of acquired immunity is 100.

Precipitin tests failed to reveal any evidence of specific tumor antibodies in either experiment.

In another group of rats from which tumors had been excised as soon as palpable, a second tumor challenge resulted in better than 95% takes.

Since it was not possible to demonstrate antibodies, Experiment 3 was carried out to determine whether cellular defense mechanisms might play a role in this acquired immunity. It seems possible that the presence of a tumor might have stimulated the hemopoietic tissues and the acquired resistance noted could therefore be explained in this way. Two substances, one inducing leukopenia (cortisone) and the other causing leukocytosis (Reticulose), were used as follows. Fifty-eight rats, 6–8 weeks old, were segregated at random into 3 groups. Animals of group 1 were given 0.4 ml. physiological saline intraperitoneally on alternate days for eight days preceding and immediately prior to tumor inoculation; group 2 individuals each received 0.25 ml. Reticulose[1] on the same schedule; and group 3 rats, 10 mg. cortisone[2] in a similar manner.

All animals received grafts from the same stock tumor. Palpation of tumors 14 days later showed (cf. Table 3) no signifi-

TABLE 3

Implantation of tumors in Reticulose- and Cortisone-treated rats

	Total Positive	Total Rats	Percent Takes	Tumor volume—mm³
Controls	21	26	80.8	313
Reticulose-treated	19	25	76	357
Cortisone-treated	17	26	65.3	485

cant differences either in number of grafts implanting nor in average attained size of tumors.

Discussion

Clear-cut demonstration of acquired immunity to tumors is difficult to obtain. Some tumors apparently elicit no immunity response while others do so. Although no typical antigen-antibody reactions were demonstrable in our experiments, it is obvious that the tumor-bearing rats were more refractory to the second implantation than the first (*cf.* Table 1), indicating some

[1]Product of Chemico Laboratories, Indianapolis.
[2]Cortisone acetate, kindly furnished by Merck & Co.

acquired immunity. Since it is possible that an "infection" immunity exists, a second experiment was designed to exclude this possibility. The results (Table 2) demonstrate that there is still increased resistance, since the same small degree of acquired immunity was evident, as had been noted in the first experiment. Again no immune factors were demonstrable serologically.

It is evident, therefore, that even though classical immunity reactions were not demonstrable, they are not wholly absent, since the acquisition of the additional immunity is dependent upon the tumor being present over a considerable time period.

Murphy (1926) believed the primary resistance forces operable against tumor implantation to be the lymphoid tissue, yet we have demonstrated that a substance, desoxypyridoxine, which causes hypoplasia of lymphoid tissue, does not significantly affect implantation (Loefer, 1951).

Table 3 presents data showing the lack of effect of two substances known to alter the hemopoietic activity. Cortisone, which elicits a lymphopenia and a depletion of lymphoid tissue (Crabb and Kelsall, 1951), and Reticulose (Thompson, 1952), a splenic extract consisting of a lipoprotein-nucleic acid complex, which causes a leukocytosis, produced no effect. Were the peripheral leukocytes influential in preventing implantation, some effect would have been expected.

It is necessary to visualize briefly the course of events surrounding and following the inoculation of a tumor fragment. The immediate response is an infiltration of leukocytes, mostly neutrophiles, and a congestion of the area, limiting mobility of elements. A great deal of serum, lymph and other highly nutritious material accumulates. Since nutritional factors are not limiting, the success of the implantation will depend therefore, upon what rapidly mobilizable defensive forces the host can elicit and upon factors which might immediately affect these, *e.g.*, ionizing radiations and pyridoxine deficiency. The forces are maximal following trocar inoculation, since additional traumatization does not enhance resistance (Loefer, Mefferd and Nettleton, 1952).

We have also demonstrated that certain non-specific treatments may significantly alter implantation, and that age of host (Loefer and Gilles, 1951), and age of animal bearing the stock

tumor (Loefer, 1952), are roughly correlated with those barriers to infectious disease which accompany host maturation (Sabine and Olitsky, 1938). As Sabine (1941) demonstrated for infectious disease barriers, we have demonstrated a striking influence of nutritional supplementation upon resistance to implantations (Loefer, 1951; Loefer and Gilles, 1951a; Loefer and Mefferd, 1952; Mefferd and Loefer, 1952a).

Resistance to tumor implantation is thus a relative factor depending upon many variables such as size of inoculum, age, and significantly, the nutritional state of the host. There is a more complex relationship between innate resistance and nutrition than is generally recognized (Mefferd and Loefer, MS.).

In animals supporting four simultaneously inoculated tumors, it was shown (Mefferd and Loefer, 1952; Loefer and Mefferd, 1953) that there was no determining influence of one tumor on another, except in a highly susceptible strain or where the tumor-host weight ratio became small, *i.e.*, tumors were very large.

It seems evident that the expression of total tumor resistance is of a very broad nature and involves many components other than humoral or cellular forces. It would appear that this problem offers a unique opportunity for the study of natural resistance phenomena.

Summary

Acquired resistance to a transplantable fibrosarcoma in Sherman-strain rats was demonstrated. A series of experiments, in which tumors were transplanted to animals bearing tumors, and to susceptible animals from which tumors had been excised, indicated that there is a degree of acquired resistance to a second tumor challenge developed in certain individuals. Precipitin tests, however, failed to reveal any evidence of specific tumor antibodies.

Groups of rats that had been treated with cortisone or Reticulose to alter the condition of the hemopoietic tissues, exhibited no significant differences either in number of grafts implanting or in average attained size of tumors. It seems unlikely, therefore, that acquired resistance is effected by means of a continuing stimulation of reticulo-endothelial tissue. The nature of the acquired resistance is not understood.

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NASAL MYIASIS OF MAN DUE TO THE SCREWWORM FLY
(*Callitroga americana*, C & P)*

DON W. MICKS AND VICTOR C. CALMA

Myiasis, a condition produced when dipterous fly larvae invade the tissues and organs of man or animals, occurs more frequently in warmer climates. The incidence of this disease may be correlated with the density of the fly population as well as with the economic status of the individual. More than one hundred different species of fly larvae are capable of causing myiasis of the nose, mouth, ears, eyes, anal region, vagina, bladder, urinary passages and gastro-intestinal tract as well as the creeping and furuncular types of dermal and subdermal myiasis (James, 1947). From the standpoint of pathogenicity, however, the most serious myiasis producers are those to which the parasitic habit has become obligatory since these fly larvae feed and develop only in living tissue.

The primary screwworm *(Callitroga americana)* is one of the more important members of this group of obligate parasites and is the most serious myiasis-producing calliphorid in the New World according to James (1947). This species was involved in approximately 90 per cent of the total number of cases (901) of wound myiasis of man and animals reported from the southeastern, middle western and southwestern sections of the United States by Knipling and Rainwater (1937). The adult screwworm flies are attracted to any sore or wound of an animal however small in which they deposit their eggs. The developing larvae inflict serious injury which often results in death in a matter of days. Though the screwworm is primarily a livestock problem, it is of more serious concern when humans are also attacked by the fly. During a severe outbreak among livestock in the South in 1935, 55 cases of screwworm infestation

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of man were reported and there were probably twice as many unrecorded cases according to Dove (1937). This species is of particular concern in a few areas such as South Texas where it occurs the year around. Infestations of the nasal region as well as the eyes, ears and mouth may result in death. Although a few investigators report a mortality rate as high as 50 per cent it appears to be much lower on the average. Thirty-nine cases of human myiasis including those in Arizona reported by Yount and Sudler (1907) were recently reviewed by Emmett and Siverly (1951) who recorded a 13 per cent mortality. All the deaths occurred in nasal infestations which comprised 77 per cent of the cases. Taylor (1950) and Aubertin and Buxton (1934) reported similar mortality rates of approximately 12 per cent and 8 per cent in their respective series of cases of nasal myiasis.

It is the purpose of this report to present three new cases of screwworm infestation of the nose occurring in Texas. Also certain discrepancies in the literature are indicated. It is hoped that this clarification may facilitate the more ready recognition and treatment of this disease since it is felt that many cases are not recognized and others are not detected early enough to be successfully treated.

Clinical Findings

Patients with myiasis of the nose and accessory sinuses frequently give a history of a fly in the nose accompanied by a tickling sensation or sneezing. More commonly, such flies are attracted to individuals who generally sleep outdoors or in quarters which are not adequately screened. Any pathological condition of the nose such as chronic rhinitis may predispose to myiasis since the foul odor which emanates from the area may serve as a very strong attractant to the screwworm flies. When the individual is sleeping, unconscious, or is unable to prevent the attack although awake, a single fly may enter and rapidly deposit a batch of from 10 to several hundred eggs in the nasal cavity. The small, first stage larvae (maggots) hatch out within 24 hours and move higher into the nasal passage. One of the earliest symptoms is a feeling of stuffiness or discomfort which is usually unilateral. As the larvae begin to feed actively, sneez-

ing may occur and the patient complains of a persistent headache often localized over one of the paranasal sinuses. At this time a sero-sanguinous discharge may be present, becoming more pronounced as the disease progresses. Around the third or fourth day there is acute local pain accompanied by swelling of the nose and face which is generally more pronounced on the affected side. The skin over the involved areas is often discolored and hot. As the larvae increase in size and the tissue destruction becomes more extensive, the pain is excruciating and the nasal discharge becomes extremely foul-smelling and purulent. Epistaxis, which is indicative of living active larvae, is usually quite mild during the first two or three days of the infestation, but later may become very extensive and difficult to control as long as the screwworms are present. A few patients may seek medical attention at this point, but many postpone it until such time as they blow or sneeze larvae from the nose or cough them up with purulent postnasal secretions. The patient's temperature usually reaches a peak of 103° to 104°. The sensorium usually remains clear, but a few patients may lapse into delirium or coma. Ocular symptoms similar to those of conjunctivitis are often present. There may be elevation of the white blood cell count to around 12,000.

Careful examination of the nasal passages will reveal a well-packed mass of active larvae partially buried in the mucosa with only their posterior ends in view. These are often difficult to remove since they grasp the tissue tenaciously with their mouth hooks, but the recovery of a larva similar to the one shown in figure I confirms the diagnosis of myiasis. This figure is a photograph of a fully matured larva removed from the patient to be presented in the following case report. At this stage the larvae are about 15 mm. long and pinkish in color.

Report of a Case

A 38-year-old colored male was admitted to the hospital with a temperature of 103° F., a pulse rate of 128 and a respiration rate of 24. The patient complained of a chronic cold for 8 years accompanied the last 5 years by a purulent foul-smelling nasal discharge. Epistaxis was noted five days prior to admission at which time he also noticed that the left side of his face about

the nose and eye were swollen. He gave a history of living in the country where he did odd jobs and had the habit of sleeping anywhere outdoors during the day or night.

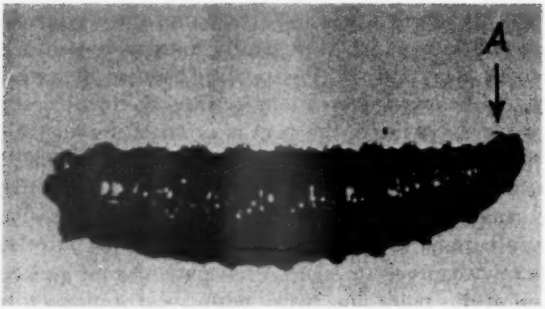

FIG. I. Mature larva of the screwworm fly *(C. americana)* removed from a nasal sinus of the patient. Note the screw-like appearance of the cylindrical, segmented larva from which it gets its name. The mouth hooks can be seen at the anterior end (A).

Upon examination by one of us (VCC) it was noted that there was a swollen area on the left side of the patient's nose. The bridge of his nose was depressed and a putrid, sero-purulent discharge was present. Screwworm larvae were present bilaterally in the nasal cavity, occurring between the remaining right turbinates and in the left ethmoid and sphenoid sinuses. The right, middle turbinate was absent as were all the turbinates on the left side. The anterior half of the nasal septum was absent and the sphenoidal sinus contained a large aperture. Fly larvae were also present in the nasal part of the soft palate. Epistaxis was also present. The laboratory examination showed a white blood cell count of 11,250 and a markedly positive (4 plus) Kolmer and Kline on two occasions.

The infested area was thoroughly cleansed by saline irrigation and cocaine (4%) was applied to shrink and anaesthetize the nasal mucosa. This was followed by a spray consisting of chloroform (5%) in olive oil and mechanical removal of the larvae. Over a period of three consecutive days of treatment, once or twice daily, a total of from 100 to 200 mature larvae were removed from the involved areas. These were identified by one of us (DWM) as *Callitroga americana.* The patient's

temperature dropped to normal within two days after treatment. Healing progressed rapidly and the patient was dismissed from the hospital one week later.

Only one of our three cases has been reported in detail inasmuch as the findings in each instance are very similar.

Discussion

As pointed out by James (1947) much of the literature dealing with myiasis needs critical re-examination in view of the numerous misinterpretations of clinical data as well as misidentifications of the fly larvae involved. The literature concerning nasal myiasis alone is quite confusing since it is full of misquotations, duplication of cases, and lack of exact identification of the etiologic agent. This may be due to the fact that those who are unfamiliar with the technical parasitological aspects of the problem often refer to outdated or incorrect information on the subject and attempt to identify the insect involved by its general appearance or location in the body. This procedure appears to be used in an article by Popov (1947) who states "The larva of the saw fly is called the Texas screwworm because of its common occurrence in the state of Texas." He cites as his main reference material six articles published between 1859 and 1898. Furthermore, two of the three presumably new cases of nasal myiasis reported by Popov (1947) had already been reported in detail by Barry (1943). The latter author classified nasal myiasis into three main divisions: (1) cavity and wound, (2) cutaneous and (3) intestinal myiasis. This classification contains entities that are not types of nasal myiasis. Five cases of the disease were presented by Barry without specific identification of the larvae. He concluded that the screwworm fly, *Chrysomyia macellaria*, is the usual etiological agent of nasal myiasis in this country. Ten years earlier Cushing and Patton (1933) had established that *C. americana* (the primary screwworm fly) and not *C. macellaria* (the secondary screwworm fly) was the species which invaded living tissue.

Marshal and Jones (1944) stated "In regions where this fly is common most cases of screwworm myiasis are seen in children - - -." Although one must not overlook the fact that children have been infested, a careful review of the literature reveals that

the majority of the reported cases have occurred in adults. Emmett and Siverly (1951), in a review of 39 cases found that myiasis was predominant in adults, 40 or more years of age.

The above constitute only a few examples of mis-statements of fact which may be encountered in a review of the literature, and which are perpetuated by those who are totally unfamiliar with the subject or review it in a very superficial manner. In order to avoid the further introduction of erroneous data into the literature it is suggested that the important publications of Hall (1948) and James (1947) be consulted. In this connection it should be noted that *Callitroga americana*, prior to the work of Cushing and Patton (1933), was referred to in the literature by a variety of names some of the most common of which were *Compsomyia macellaria* and *Chrysomia macellaria*. Inasmuch as Cushing and Patton (1933) showed that *Cochliomyia americana* and *Cochliomyia macellaria* are separate species, literature since 1933 may correctly refer to *C. americana* as a parasite of living tissues, but not to *C. macellaria*. *Callitroga* is the presently accepted genus name for these two species.

In view of the previous findings (Dove, 1937) that a relatively large number of screwworm infestations in man was associated with a heavy attack rate in cattle, it should also be pointed out that a considerable number of reported cases have occurred where the screwworm population was low and where the individual had no particular association with animals of any kind. For example, in the three cases reported by us involving two colored males 38 and 43 years of age and one Latin American boy 18 years of age, only one lived in the country where he might have been exposed to numbers of primary screwworm flies. It should be remembered, however, that the adult female fly is capable of flying at least 9 miles (Parish, 1937) and therefore close association with livestock apparently is not an essential predisposing factor.

A pre-existing pathological condition of the nose or sinuses, which was a characteristic finding in our cases as well as in reports by others, is one of the most important factors which contributes to the serious nature of nasal myiasis. This is partly due to the fact that screwworm larvae are usually superimposed upon an already diseased or traumatized tissue. Syphilis, for example, is listed as a predisposing factor by Taylor (1950)

and was present in two of our cases. Not only are the flies strongly attracted by the very disagreeable odor, but also the penetration of the larvae is greatly facilitated by previous tissue destruction and therefore a more serious condition may result. This is most clearly demonstrated in our detailed case report of a 38-year-old colored male who had tertiary syphilis resulting in a typical luetic nose. This pre-existing condition allowed the larvae to make deeper penetration toward the brain or other vital areas. Because of the inherent danger in the activity of fly larvae in this area, there is always a strong possibility of complications such as meningitis, hemorrhages and extension of the disease to the eye, ear, mouth or throat.

These findings do not preclude the possibility that the normal, nasal mucosa may become infested with screwworms. Stroud (1927) and Marshal and Jones (1944) reported cases of myiasis in apparently disease-free individuals. Wallace (1936) liekwise noted that although there is little likelihood of an infestation in a normal, healthy person; it could occur in childhood, in the aged or debilitated. It should be mentioned that although the nose is the most common site of attack, the screwworm showing a preference for this area (Marshal and Jones, 1944); the eyes (Anderson, 1935), ears (Turnbull and Franklin, 1942), (Melton and King, 1943) pharynx and mouth (Shira, 1943) may also be attacked. Not only are sites other than the nose occasionally involved, but this area may on rare occasions be invaded by the larvae of other flies (Beachley and Bishopp, 1942).

Early diagnosis is of the utmost importance but may be difficult to make during the first two or three days of the disease. This is particularly true when the patient is not aware that a fly has entered the nose and thus often postpones medical attention. In this connection it is interesting to note that none of the three cases reported by us was admitted to the hospital until approximately one week after onset of symptoms. The maturity of the larvae was further evidence of the advanced stage of the disease.

Since screwworm infested tissue has been thought by some to be heavily contaminated with a variety of bacteria, it is noteworthy that Borgstrom (1938) found that the infested lesions

in 70 laboratory cases of *Cochliomyia americana* infestations of guinea pigs invariably developed a pure culture of a single species of bacterium, *Proteus chanderli*. This organism was also found to be present as a pure culture in several natural infestations of other animals. Barry (1943) found almost pure cultures of *Streptococcus hemolyticus* in nasal smears and cultures from two of his cases. It appears that very few attempts have been made to isolate and identify strains of bacteria associated with nasal myiasis.

Several authors including Taylor (1950) have suggested that the patient should be guarded against reinfestation by other flies while the primary case is being treated. They offer no evidence that this has ever occurred and we have been unable to find any report of a reinfestation while the primary attack is still undergoing therapy. The extent to which immunity might function in preventing a second attack of human myiasis is not clear. Borgstrom (1938), however, has found that a single infestation of *C. americana* in guinea pigs produces a temporary, local immunity lasting between twenty and forty days after the primary infestation. The immune response was found to be of a local character, largely confined to the specific area infested.

Inasmuch as those who have myiasis of the nose often live in more isloated areas quite distant from the hospital, it is important that the physician be familiar with symptoms of the disease in order that it may be diagnosed as soon as possible. Treatment should be instituted immediately to prevent further damage to the tissues. Methods of treatment have been only slightly revised in recent years mainly because chloroform, which has been used for more than half a century, generally provides satisfactory results without marked, undesirable side reactions. Also, it is more readily available to the average physician than benzol or some of the other drugs which have occasionally been used. The literature is quite controversial as to the purpose of using chloroform. In order to clarify this point it should be mentioned that the initial effect is that of stimulation, causing them to release their hold on the tissue and to leave their burrows. Several minutes exposure to the anaesthetic generally kills the parasites. As a result of this effect of the drug, one is able to detect hidden larvae and to remove all visible parasites

mechanically. The most effective method of treating this type of myiasis would appear to be a modification of that suggested by Wallace (1936) and others. The nasal passages should be wiped clean and irrigated with saline to remove the secretions. Cocaine (4%) or adrenalin may then be applied to shrink the nasal mucosa, followed by treatment of the area with chloroform. Brown (1945) states that, depending upon the circumstances, chloroform may be used undiluted as a spray, as an irrigation in the form of a 50% solution or as 15% solution in a light vegetable oil. In addition to employing 25 to 50% chloroform as a nasal douche, Barry (1943) recommends using it full strength (USP), applied by means of saturated cotton pledgets inserted into the affected area. A review of the literature indicates that the application of 15 to 25% chloroform in a light vegetable oil as reported by Emmett and Siverly (1951) is the treatment of choice. The stunned or killed larvae should then be removed mechanically by the use of forceps. This routine should be followed two or three times daily until there is no further evidence of the presence of living screwworms. Appropriate measures should be taken to relieve the pain and maintain a satisfactory nutrition. In the case of children, chloroform anaesthesia will enable one to secure the desired results (Stroud, 1927). In the treatment of secondary infection, any of the wide-spectrum antibiotics might be used; however, in view of the findings of Borgstrom (1938) better results might be attained by the use of an antibiotic which is more active against gram-negative organisms. It is suggested that where possible nasal cultures would be helpful in the intelligent treatment of the secondary infections.

There are a number of preventive measures such as those outlined by Pearman and Haseman (1943) which could be employed to very good advantage. These consist of (1) protection of all wounds, especially those of the nose and ears from flies; (2) treatment of chronic conditions of the eyes, ears, nose, mouth and throat, and avoiding sleeping outdoors while these pathological conditions exist; (3) complete screening of living quarters; and (4) control of flies both by direct application of insecticides and the proper disposal of screwworm-infested carcasses. The latter measure should receive first attention with the employment of an insecticide as only an auxiliary measure.

Summary

Three new cases of nasal myiasis from Texas are reported. The pertinent literature relating to predisposing factors, symptomatology, diagnosis, treatment and prevention of nasal myiasis is reviewed in an effort to clarify some of the conflicting reports therein.

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CENTRAL HEATING SYSTEMS AND ASSOCIATED POTENTIAL DANGERS OF CARBON MONOXIDE*

CARL A. NAU AND JACK NEAL

With the general availability and low cost of natural gas, this fuel is used extensively for heating our homes. Such heating is convenient and clean. Natural gas is largely methane and contains no *carbon monoxide* as it is supplied to the user. Death associated with the use of natural gas is the result of asphixiation either as a result of displacing the air with its available oxygen with the methane without oxygen; or it is the result of incompletely burning the natural gas due to improper mixtures of gas and air and thereby generating carbon monoxide which combines with the hemoglobin and produces asphyxia as a result of such immobilization.

Carbon monoxide is generated when any fuel is incompletely burned—be the fuel coal, oil, wood, petroleum, or gas. It is an important component of manufactured gas and of the exhaust gas of automobiles and other combustion engines. But it is *not* found in natural gas.

Because natural gas is so accessible, so cheap, and so convenient it is used as a fuel for individual space heaters. These heaters are moved from room to room, are rarely ever checked or adjusted, and if they are adjusted, they are easily "thrown out of adjustment." This may happen when the heater is moved or when it is "hit" by a sweeper or chair when house cleaning is done.

Assuming the space heater is properly designed, constructed, approved, and maintained, it may not "put-out" any carbon monoxide under the usual and normal usage. But when such heaters are first lighted in a cold room and the gas jet is open wide, complete combustion rarely occurs until the radients are "red-hot"; and during this interval, carbon monoxide may be generated.

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Assuming that no carbon monoxide is generated in significant quantities, space heaters are still objectionable because: (1) They are usually connected to the gas jet by a rubber tube which is rarely ever checked for leaks, (2) They are often connected to the gas jet by copper tubing which cracks easily when hit by a sweeper or chair, etc., and is rarely checked, (3) There is generated and dissipated into the room a large amount of water vapor—coming from the combustion of the gas. To this is added the moisture added by the inhabitants of the room through perspiration and/or respiration. The room air is saturated with this water vapor rather quickly and the water condenses on all cool surfaces—such as, windows, lavatory, and other bathroom fixtures, cool walls and clothing and shoes in closets. The high humidity makes the room air seem "stuffy" and objectionable.

These many factors have led to the present trend toward "central heating plants" where one large vented heater is used to heat the air and the heated air is forced through ducts by a motor driven fan into the various rooms of the home or building. The air from these various rooms is returned to the heater, re-heated, and re-circulated. In the process, varying amounts of fresh air may be brought in from the outside and mixed with the re-circulating "used" air.

Obviously, the less cold air that is brought in to mix—the cheaper the cost of operation. Such attempts to "cut costs" takes no account of the changes in O_2, CO_2, and moisture content of the re-circulating air. The general practice is to supply the burner of the heating unit with a portion of the re-circulated air for air–gas mixing. Rarely ever does the burner have its own supply of fresh air from the outside. Should the O_2 content of the re-circulating air fall appreciably, the burner would become less efficient and CO would "come off" in the stack gases in varying and increasing amounts.

In general, the return air to the heater has to "find its way back" through louvers and grills in doors and walls; and it is assumed that there is enough fresh air brought in by seepage through cracks in doors, windows, etc., and/or through the opening of doors in the normal home living. Often this factor of seepage is overemphasized; especially, when homes are weather stripped and walls and ceilings are insulated.

Few cities have satisfactory ordinances covering design, construction and/or installation of central heating systems. Even if they have such, inspection and enforcement of such ordinances is rare. At times, inspections are made by untrained inspectors. Often, even contractors are poorly informed and/or disregard health and safety precautions for the sake of a few extra dollars of profit. The owner or buyer of the home rarely has any knowledge concerning the potential hazards of a central heating system—as many of them are being installed (or exist) today.

As a result of such ignorance and/or carelessness, central heaters are often installed in small closets—frequently in the same closet where the hot water heater is installed. The air returning to the heater from various sections of the home has to "find its own way to the heater" through a grill in the closet door or wall. Frequently this returning air reaches the heater through the closet itself from which the burner also gets its air for mixing with the gas for combustion. We have seen many such installations (a few with tragic consequences) which we will briefly describe.

Figure I. A 100,000 B.t.u. heater in a small closet in which a 30-gallon hot water heater (natural gas) is connected. Both are vented through separate vents through the roof. The returned air for reheating enters the closet through a louvered door (7′ tall and louvered from top to bottom). The air "feeding" the burner of the central heater and also the hot water heater has to enter the closet through the louvered door. Both heaters have a stack diverter on their respective vents in the closet. The fan drawing the air through the fire tubes for reheating operates only while the main flame in the heater is burning and for about a minute after the flame is shut off.

Under normal circumstances, the louvered door would permit the entrance of adequate return air to meet the requirements of the fan and the burners of both hot water and central heater. But, if a paper should fly against a portion of the louver or through some other accidental or willful act, a portion of the louver were obstructed, then the fan could no longer draw enough air through the louvers and would satisfy its needs by "sucking" air from either or both stacks through the respective draft diverters. These stack gases contain CO which would then

be drawn into the duct system and be distributed to all sections of the home. The "partial" negative pressure set up in the closet because the heater fan could not draw in an adequate supply of air through the louvered door would also lead to an inadequate supply of air (and oxygen) to the burners. Hence, the stack gases might contain an increasing percentage of CO.

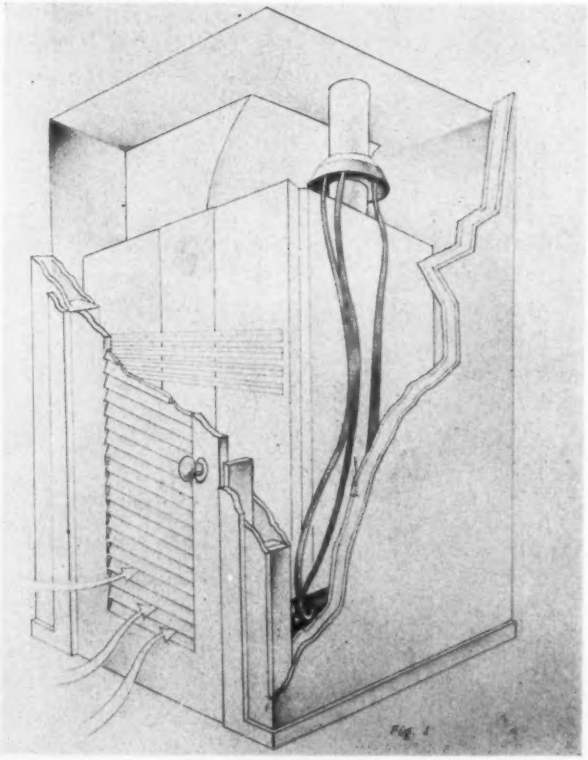
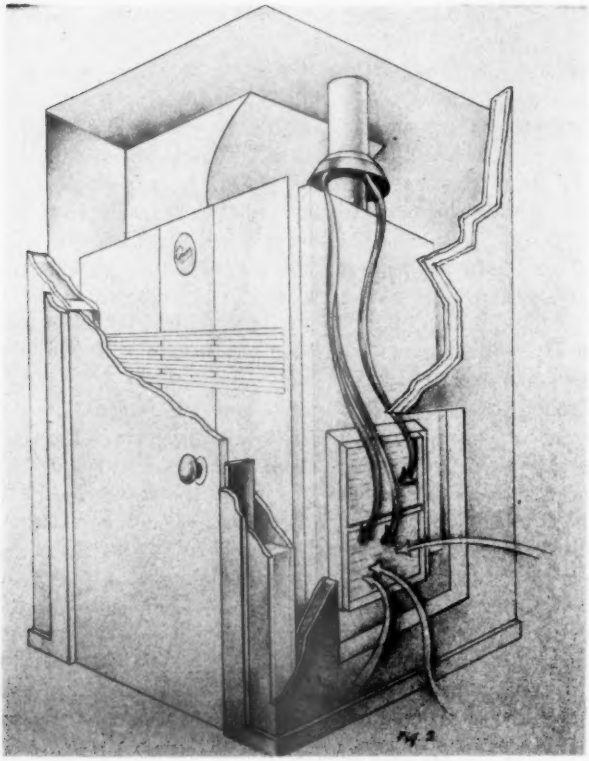


Figure II. A 100,000 B.t.u. heater in the small closet with closet door unlouvered and opening into the kitchen. The return air entered the heater closet through a grill in the closet wall from the living room. There was no direct and "tight" connection from the living room to the return in the heater. In fact,

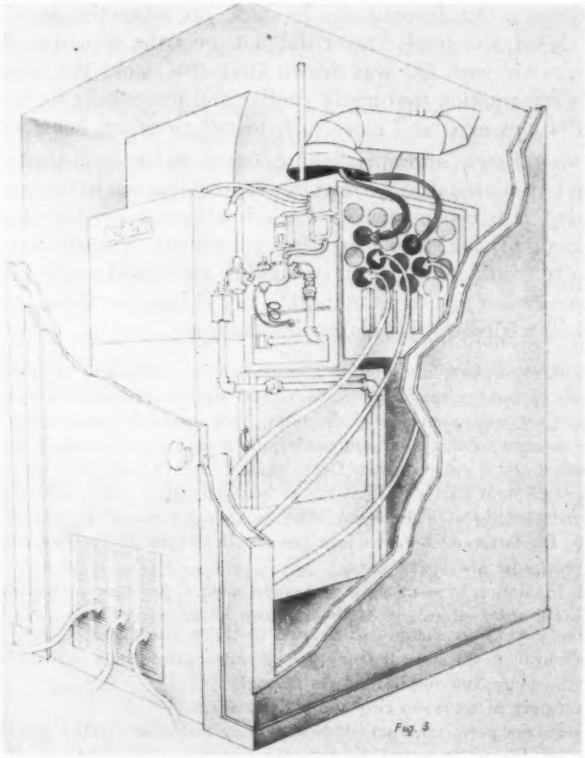
the return into the heater was about 6 inches from the grill. Therefore, the heater was actually getting its air from the closet. If the grill to the living room were partially or completely obstructed, a negative pressure could be created in the closet when the fan was running and air "could be sucked" from the stack through the stack diverter. Actually, when the unit was inspected a console radio was sitting in front of the grill.



The owner of the home (a responsible business executive) stated that he always left the heater closet door open. But on another inspection, the closet door was found closed. Of course, it is obvious that a "man of the house" can never be certain

whether the closet door will be open or shut; and particularly if an unsightly view is evident with the door open. The burner of the heater also got its air for combustion from the closet. It had no outside fresh-air supply.

Figure III. A 100,000 B.t.u. heater in a small closet together with an air-conditioning cooling unit. The closet door opened



into a hallway and had a 15″ x 15″ louver. The fan ran constantly. All of the return air as well as the air to the burner had to come into the closet through the door louver. Obviously, this was inadequate and hence stack gases were drawn back through the diverter and mixed with the return air going to the duct dis-

tribution system. Within 30 minutes after the main heater fire was burning, CO in excess of 1,000 ppm. was in every room of the home. As a result, the first night when the system was used, 5 members of the family were found unconscious the next morning at 8:00 A.M., and one member of the family (3½ year old boy) died the same day. It was obvious that the heater had been incorrectly installed. When the closet door was left open, there was no air drawn from the stack; but when the closet door was closed, the small louver did not meet the demands of the fan and air with CO was drawn from the stack. We have reliable information that many similar and potentially hazardous installations exist and more such installations are being made.

It would seem appropriate to comment on the treatment often given to individuals overcome by CO as compared to that recommended. It is not uncommon to find patients given transfusions of blood, plasma, saline or other substitutes. Transfusions are actually considered to be harmful. The treatment approved and recommended by the Committee on Poisonous Gases of the American Medical Association is as follows:

(a) If respiration has stopped, apply artificial respiration by the prone pressure method immediately. Mechanical devices for artificial respiration are not recommended as substitutes. This should be commenced without a moment's delay and continued until spontaneous breathing returns, or until death is fully proved. Death should never be admitted until artificial respiration has been used for at least one hour, while recovery has occurred as long as eight hours after persistent artificial respiration was begun. Death should only be pronounced after repeated examinations fail to reveal pulse and heart sounds.

(b) Inhalation of a mixture of oxygen with 7 per cent carbon dioxide as a respiratory stimulant. If the mixture is not available, oxygen alone may be used. This should be initiated in the shortest possible time after the removal of the patient from the poisonous atmosphere and continued until the pulse and respiration are normal.

(c) Apply measures to keep the patient warm.

(d) Do not permit the patient to make any muscular exertion until fully recovered. Do not transport the patient a distance.

(e) Refrain from hypodermic medication or transfusion of blood.[1]

If carbon monoxide is suspected of being the cause of symptoms of illness or death, a drop or two of blood (or, if death has

[1]Boyd, Mark F. *Preventive Medicine*, Sixth Edition, Revised, W. B. Saunders Company, page 433, 1941.

occurred, a small clot of blood) should be submitted to a competent analyst. He can quickly determine the presence or absence of carbon monoxide—hemoglobin and the percentage of hemoglobin so "tied-up," even though death may have occurred days ago.

Conclusions

1. Central heaters (properly designed) are often incorrectly installed and through such faulty installations may be potentially hazardous.
2. The return air to a heater unit in a closet should be from another part of the house through a "tight" and "fixed" connection and not through the closet door.
3. The burners should get their air for air-gas mixture from an easily accessible source—preferably the outside or from 2 openings in the door to the room holding the unit—one louvered opening adequate in size at the top of the door and a similar opening at the bottom of this same door.
4. All installations and/or changes in installation should be inspected before use by a competent and effective inspector.
5. All cities should have ordinances to cover heater installations with the necessary "machinery for enforcement of such ordinances."
6. Treatment of people affected by CO should follow the recommendations of The Committee on Poisonous Gases of the American Medical Association.
7. No transfusions are indicated. In fact, they are potentially dangerous.

AN AUTOMATIC DUST FEED APPARATUS USEFUL FOR EXPOSURE OF ANIMALS TO DUSTY ATMOSPHERES*

CARL A. NAU, JACK NEAL, AND A. P. FREUND

Some years ago (1943), one of us reported an apparatus useful to expose small animals to small and fixed concentrations of dust (1). We used this set-up to study the effect of inhalation of antimony oxide and silica gel dusts and also, early in our study, on the physiological effects of carbon black dusts. In the course of these latter studies, however, we saw the need of making certain changes in design and construction; such changes giving us a more automatic and more easily controlled device.

It has always been necessary to control humidity and temperature. Whereas, early we accomplished this by cooling the air in our dust chamber, we now accomplish it by air-conditioning the air in the larger laboratory from which a large portion of the air supplying the dust room is obtained. The compressed air supplying the primary jet for the dust pick-up is cooled by passing it through a copper coil cooled by the air-conditioned air in the larger laboratory.

The present equipment has been used for one year and, in our opinion, lends itself well for use in creating dust concentrations which can be kept at the same level for years without any significant fluctuations. The set-up also permits us to maintain a uniform temperature and humidity. The equipment is adequate to make sufficient "dusty air" to supply a room 6′ x 10′ x 8′ in which various species of animals can be exposed. We now use mice, guinea pigs and monkeys.

The dust-laden air from the exposure chamber is conducted from the chamber to a dust bag placed in a dust house having two dust-tight compartments. The bag is shaken down at frequent intervals into a drawer in the lower compartment. This arrangement is more effective than the method of electrostatic precipitation used previously.

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*From the Department of Preventive Medicine and Public Health and the Technical Apparatus Shop, The University of Texas Medical Branch, Galveston. Received for publication August 1, 1952.

Dust sampling is done from various parts of the chamber through port-holes. Such repeated sampling at varying intervals of time reveals the presence of concentrations of dust which are practically identical regardless of the temperature and humidity of the external air.

Apparatus

The difficulties we encountered in using the apparatus previously described were:

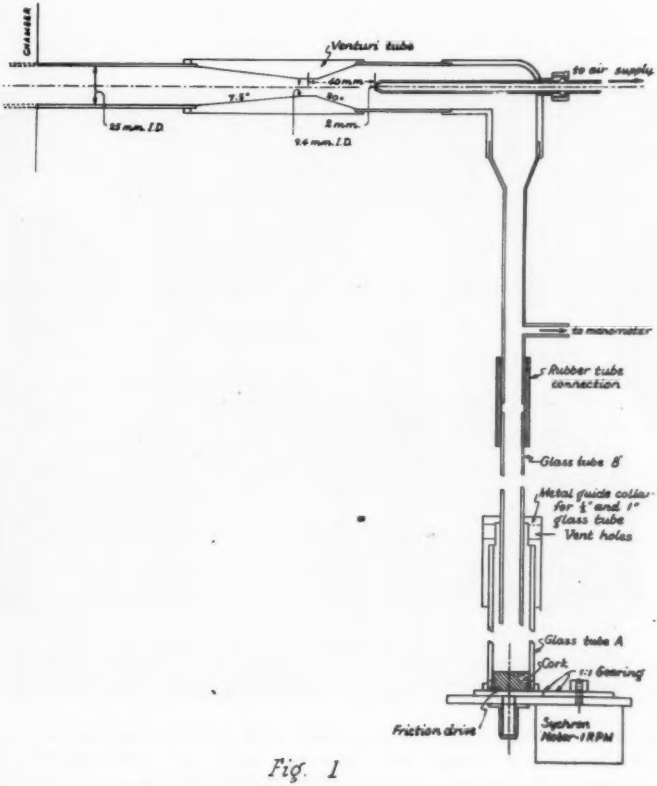


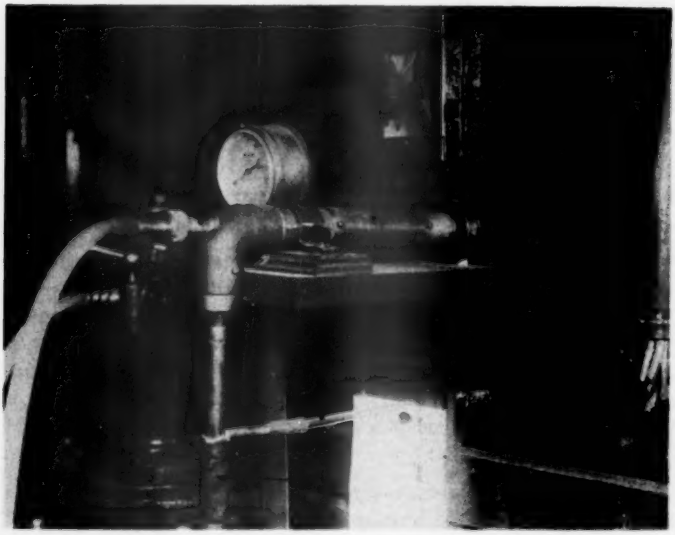
Fig. 1

Cut-away section of venturi and dust reservoir tube.

1. Clogging of the venturi tube.
2. Lack of uniform elevation of the dust column.
3. No automatic stoppage of the elevator when the dust column was depleted.
4. Lack of uniform rotation of dust tube.
5. Elevator speed changing mechanism difficult to adjust.
6. Trapping carbon black with an electrostatic precipitator.

To eliminate these difficulties several basic changes were made:

1. The clogging of the venturi tube and orifice was overcome by accurately machining the venturi and its component parts from brass tubing (see Figs. I and II). The same formula for the throat and orifice was



Venturi ejector mounted on dust chamber.
8—venturi tube. 9—air inlet. 10—to manometer. 11—air reduction valve. 12—rubber tube connection, for easy assembling and disassembling.

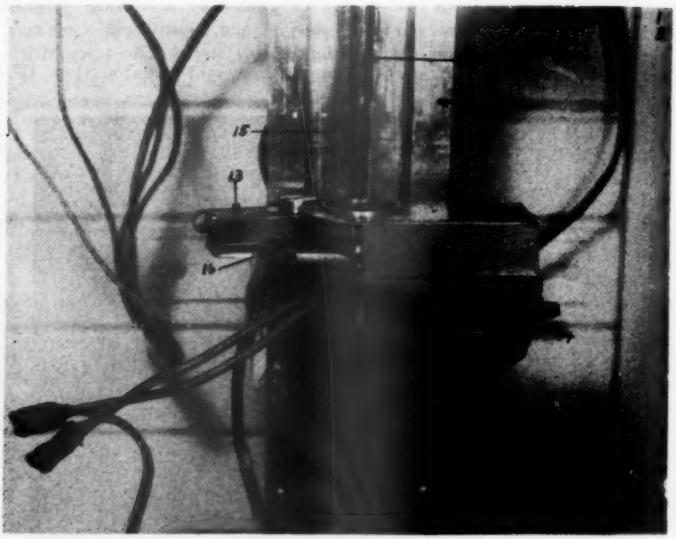
used as described in the previous article with the exception that the point of entrance was changed to a right-angle bend with orifice tube located exactly in its center (see Fig. I). The cyclone dust sizer and the brass spiral with wire spikes as shown on page 91, figure I, of the March, 1944,

article were eliminated completely. As a result of these changes, carbon blacks do not clog the venturi or dust tube even after several 8-hour days of operation.

2. The lack of uniform motion was eliminated by machining a brass carriage for the dust reservoir to elevate on and substituting a positive screw motion drive for the old wire and pulley set-up. The screw drive and carriage is shown in figures III and IV. A split nut stop (Fig. IV, No. 13, and Fig. V) has been devised to provide means of returning the elevator to the bottom of the carriage after each 8-hour run.

Front view of complete dust feeder mounted on dust chamber.

3. An automatic switch is built into the elevator guide to provide a positive cut-off at the end of 8 hours of operation (Fig. IV, No. 15).



Front view of dust reservoir "rotating-and-return" mechanism.

5—3/8" x 16 threads per inch elevating screw. 7—main frame which supports all of the mechanism. 13—nut release lever. 14—1 r.p.m. Synchron motor. 15—automatic shut-off switch. 16—gear, 1 r.p.m. driven by Synchron motor 14, this gear has recess for driving 1" glass tube which contains carbon black.

4. The motor-driven flexible cable of the older apparatus has been replaced with a direct connected synchron motor, shown in figure IV, No. 14, so that a uniform rotation of the dust tube is assured.

5. Speed changing in the old device was very difficult to adjust due to slippage and stretching of the lifting wire. This has been changed by machining three sets of gears and attaching a selector arm (Figs. VI and VIa) for setting of speeds for lifting the elevator 24 inches, 16 inches, or 8 inches in 24 hours—thus enabling one to change the dust concentration by merely setting the selector to a higher or lower lifting speed.

6. The electrostatic precipitator dust collector as described in the March, 1944, article was not efficient enough for trapping all of the carbon black after it had passed through the dust chambers. Thus, it was necessary to design a bag house and select a cloth that would not clog with black in

a short time. This was done by selecting a coarse 8 ounce cotton duck cloth and letting the carbon black form its own filter on the surface of the bag. The bag shown in figure VII has been in constant operation for four months without replacement. A weekly shaking-down of the bag is all of the upkeep necessary on the bag.

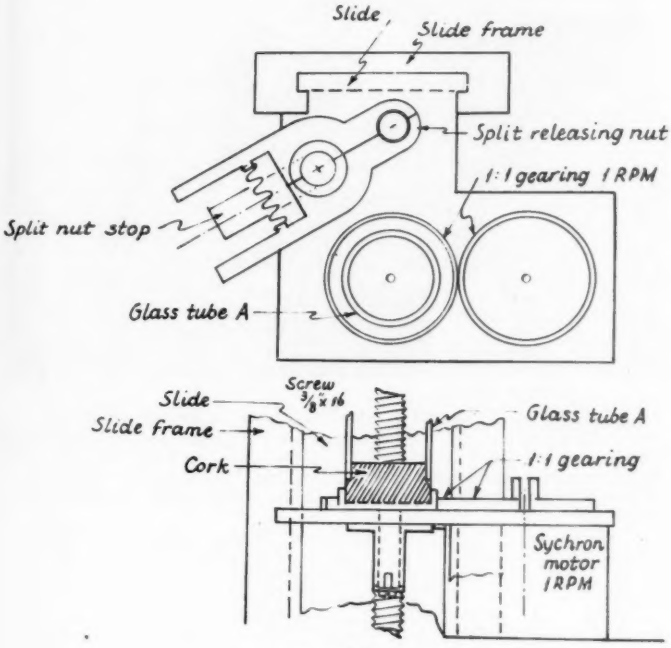
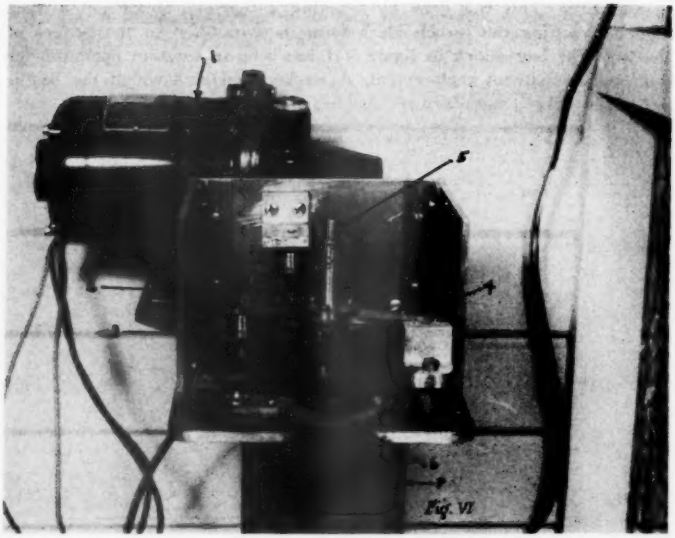


Fig. V

Top view of split-nut stop and front view of dust-reservoir-rotating-mechanism.



Front view of speed selector and reduction geared motor.

1—1/75 h.p. motor. 2—3-speed cluster gear. 3—Idler gear, in mesh with cluster gear and gear on screw 5. 4—Idler adjusting lever. 5—3/8″ x 16 threads per inch elevating screw. 6—Hock for 1″ glass tube which contains carbon black and rotates 1 r.p.m. 7—Main frame which supports all of the mechanism.

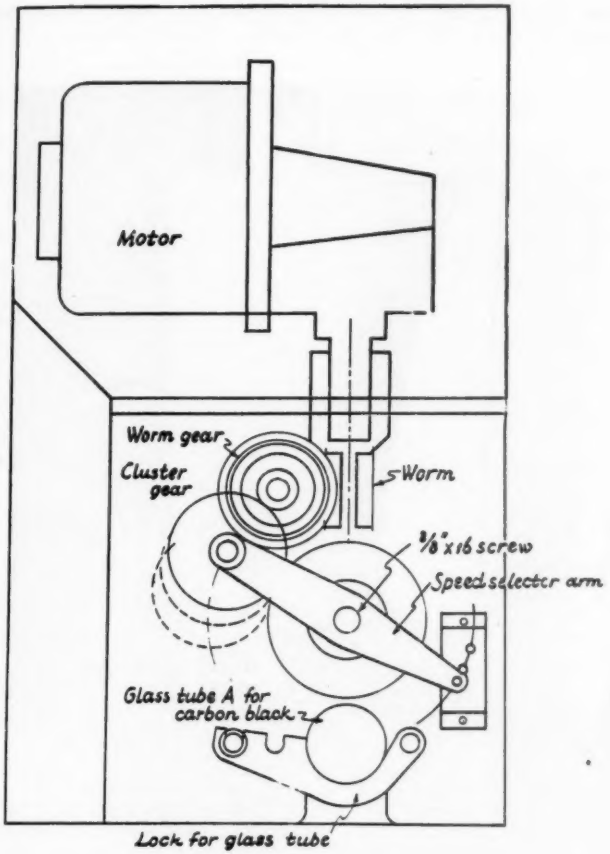


Fig. VIa

Top view of speed selector and motor.

The changes in the dust feed apparatus as described have proven meritorious. We now have a set-up which is capable of reproducing identical conditions of the air day after day; is easy to maintain; and has incorporated in it a number of automatic controls. It can be used to create dusty air for experimental

studies involving any of the more commonly used laboratory animals.

Front view of bag house and filter with shake-out compartment open.

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THE TEMPORARY SURVIVAL OF TRYPANOSOMA EVOTOMYS IN TRIATOMA*

A. Packchanian

In previous communications, it has been shown that *Trypanosoma brucei*, *Tr. duttoni*, *Tr. lewisi*, *Tr. gambiense*, *Tr. hippicum*, *Tr. rhodesiense*, and *Leishmania donovani*, and *L. tropica* will not survive in *Triatoma* more than ten days (Packchanian, 1948). The present study adds another species of trypanosome which will not survive more than five days in *Triatoma gerstaeckeri*.

A White Mountain red-backed mouse *(Evotomys gapperi ochraceus)*, captured in Rutland, Vermont, was found to be naturally infected with *Trypanosoma evotomys*. The flagellates in the circulating blood of this mouse were small and narrow, resembling *Tr. duttoni* (Wenyon, 1926). This mouse was used as a host for *Triatoma gerstaeckeri* in order to determine whether or not *Tr. evotomys* will survive or multiply in this insect.

Eleven normal, laboratory-reared *Triatoma gerstaeckeri* nymphs were allowed to feed on the naturally infected red-backed mouse *(E.g. ochraceus)*. At the time of feeding, the circulating blood of the mouse contained many active trypanosomes. Four days after the infective blood-meal, the fecal material from one of the *Triatoma* was examined microscopically with negative results for active flagellates. However, when this insect was crushed and the fecal material was examined, one sluggish and several dead trypanosomes were noted. Similar examinations of eight additional nymphs 18 and 20 days following the infective blood-meal all gave negative results for viable trypanosomes.

The two remaining nymphs, 25 days following the infective blood-meal, were crushed in saline and the suspension was inoculated into a normal red-backed mouse *(E.g. ochraceus)*. The

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peripheral blood of this mouse was examined at various intervals up to 49 days, with negative results. At the end of the 49th day, the heart blood of this mouse was inoculated into N. N. medium and incubated at 25° C. Susbsequent examinations of this culture, during two to four weeks of incubation, were all negative for flagellates.

It is concluded, on the basis of microscopic examinations, cultural, and animal inoculation tests, that *Tr. evotomys* will neither multiply nor survive more than five days in *Triatoma gerstaeckeri*.

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DYNAMIC NEUROGLIOLOGY*

C. M. Pomerat

We are meeting today not only to dedicate the new Gail Borden Laboratory which will house our departments of biochemistry, physiology, pharmacology and microbiology but also to honor the alumni of this school who have spent twenty-five and fifty years in the practice of medicine. As I look into the twinkling bright eyes of these youngish old graduates, I cannot but feel the timelessness of the human element in the life of science. I see no essential difference in the inquisitive look of "old grads" from that which I see in our present medical freshmen. There is the same quality of independence, curiosity and mischievousness as has always animated the look of men and women who are deeply interested in the phenomena of life and death! I would assure our graduates, too, that the same quality of timelessness is to be found in the outlook of old and new professors! The faculty today is to the present freshman class the same overbearing group of taskmasters as they were in your day! They are also just as curious about, and dissatisfied with, the present state of our limited knowledge regarding the so-called "mysteries of the living organism" as were the men you knew when you were in medical school. In spite of cumulative mountains of textbooks, monographs and original research articles, there still are obscure fundamental problems which are as inviting challenges to the present faculty as they were twenty-five and fifty years ago.

Another anniversary is being celebrated this year. The father of neurology and one of the great architects of modern medicine was born one hundred years ago. The themes which obsessed Ramón y Cajal, the pattern and the interplay of elements in the nervous system, remain a rich continent for present day explorers. In spite of the magnificent contribution which Cajal and his followers made to our understanding of neuroarchitec-

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*From the Tissue Culture Laboratory, The University of Texas, Medical Branch, Galveston, Texas. Presented in part at the dedication of the Gail Borden Memorial Laboratory of the Medical Branch at Galveston, June 6, 1952, and in honor of Santiago Ramón y Cajal on the centenary of his birth.

tonics, we are finding ever-fresh approaches and opportunities for inquiry into this passionately exciting system of cells.

Following upon the pronouncement of the neuron doctrine, it was altogether natural that interest in nervous function should have been focused almost exclusively on the neuron itself. The prominent role of neuroglia in pathological processes and the suspicion that the failure of nerve regeneration in the CNS might be due to glial reaction invited the attention of numerous experimentalists but who generally were not seriously concerned with the possibility that efficient neuronal metabolism under so-called "normal" conditions might be dependent upon the activities of astrocytes, oligodendroglia and microglia. In spite of general neglect regarding the dynamic role of neuron-associated elements, authors of textbooks of neurology and the writings of practically every serious student of nervous function have hinted at their probable importance. For example, after citing Bok's (1936) finding that neurons occupy only about 2.85% of the total volume of the human cortex while the remainder is composed primarily of glia, Gerard (1950) wrote: "Conversely, the tendency to relegate glia to a casual structural role in the nervous system is surely myopic. I suspect these cells are important in nutrition—though the 'sucker-foot' approach leaves much to be desired—and I know of no evidence that excludes them from a role in neural function, even in impulse conduction. It is reported by Kuntz and Sulkin (1947), rather, that preganglionic stimulation leads to glial hypertrophy in autonomic ganglia. Dr. Tschirgi, who holds similar views, is, accordingly, initiating an intensive study of the glia from the physiological viewpoint. Important activity of glia in pathological changes in the central nervous system, by contrast, is well known."

My own interest in the role of neuroglia was awakened as a result of the preparation of phase contrast, time-lapse cinematographic records of the behavior of cells from the adult human cerebral cortex. Some four years ago, a program was set up here for making tissue cultures of biopsies of brain tissue obtained in the course of psychosurgical procedures. With the aid of Professor I. Costero of the University of Mexico, this study was soon enlarged to include intracranial tumors. During a visit to London

in 1949 the observation was discussed with Dr. Dorothy Russell of the London Hospital that moving picture records showed pulsatile activity in cells from a subject lobotomized for intractable pain resulting from a malignancy involving the upper respiratory tract. Professor Russell recognized the relation of this phenomenon in nonmalignant brain tissue to similar observations made by Canti, Bland and Russell in 1935, of pulsating cells in tissue cultures of an oligodendroglioma. Subsequent investigations led to the publication of a report describing rhythmic activity in oligodendroglial cells from the corpus callosum of the normal rat by Lumsden and Pomerat (1951) and a summary of cinematographic observations upon pulsatile and contractile cells in human brain material (Pomerat, 1951a).

While the presence of cells in brain explants showing rhythmic movement yielded striking records, acceleration of cellular mobilities also demonstrated extraordinary activity in membranes along the course of astrocytic processes. These observations are no less important in the construction of a dynamic concept of brain structure.

The magnificent cinematographic studies by R. G. Canti, W. H. and M. R. Lewis, G. O. Gey, H. B. Fell and many other workers utilizing the tissue culture method, as well as the records made *in vivo* by Speidel, have done much to "decondition" the static concepts which we acquire in the course of studying cells and tissues with the classical techniques of the fixed preparation.

The present discussion is designed to re-emphasize the importance of interpreting the vast accumulation of information regarding the cytology of nervous tissue obtained from metallic preparations in terms of the probable appearance and behavior of the corresponding living cells. This point can be illustrated with a brief consideration of microglia. Textbook descriptions of microglia usually state that these cells have ". . . two, three or more spiny frequently branching processes." (Ranson, 1939). Students are taught that in ideal preparations such cells often present a "thorny" appearance. Typical examples have been reproduced from Rio-Hortega's (1949) drawing in figure 1. By way of contrast, photomicrographs in figures 2–10 represent the appearance of microglia as seen in the living state at half-

minute intervals taken from a moving picture record. It will be noted that the twiglets of the so-called "branches" actually are very delicate membranes. Indeed, even with the aid of phase contrast microscopy it is almost impossible to define the limit of the membranous processes except when they are seen edgewise. Cine-photomicrographs taken at a rate of 8 frames per minute and projected at 16 frames per second(acceleration of 120 x) show that the "spiny processes" of such cells actually indicate the location of multiple delicate membranes whose movements may be described as resembling a series of puffs of smoke arising from multiple points. Studies of these paucimolecular extensions of the cytoplasm make it easy to understand that while they may be reasonably well preserved by the rapid fixation of tissue culture preparations, where the cells are free and in a thin plasma clot, the usual methods of histology produce retraction of the multiple membranes in linear aggregations of cytoplasm which attract the deposition of silver in such a way as to form branching "twiglets." The discrepancy between textbook illustrations of microglia with what we can see in the pattern of living cells with phase contrast, time-lapse cinematographic records and the intermediate picture obtained by rapid fixation of extremely thin tissue culture preparations led us to undertake studies on the effect of fixatives as generally employed in neurocytological work upon nervous structure.

Figure 11 illustrates a device in which cells can be examined microscopically while fluid is perfused through an inflow tube (a), across the thin space occupied by the tissue culture preparation (b), and out of an exit capillary (c). Such perfusion chambers are utilized for direct study or recordings either with "still" photomicrographs or with a cinematographic setup (Fig.

PLATE I

FIGS. 1–10. Microglia.

FIG. 1. Shows microglia in brain tissue as revealed with classical neurohistological techniques. From a drawing by Rio-Hortega (1949).

FIGS. 2–10. Living microglia as seen with time-lapse, phase contrast cinematography. Frames were selected from a 16 mm. film to show changes in ruffled membranes at 30-second intervals. The cells were found in the outgrowth of a 7-day culture of a metastatic melanoma from the frontal lobe of a 42-year-old white male (H. McC., J.S.H. No. 2567-H).

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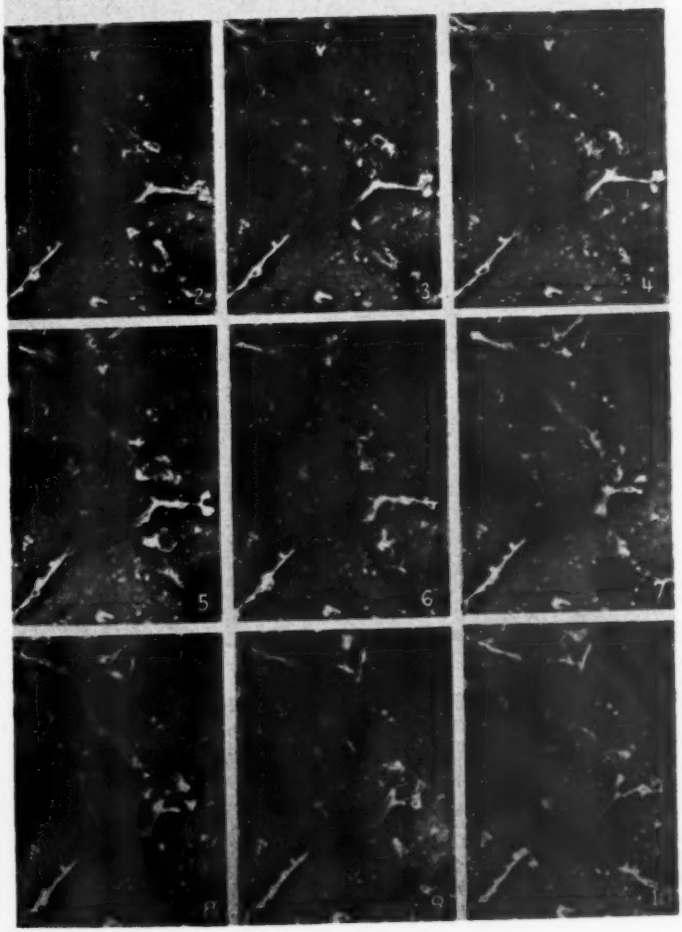
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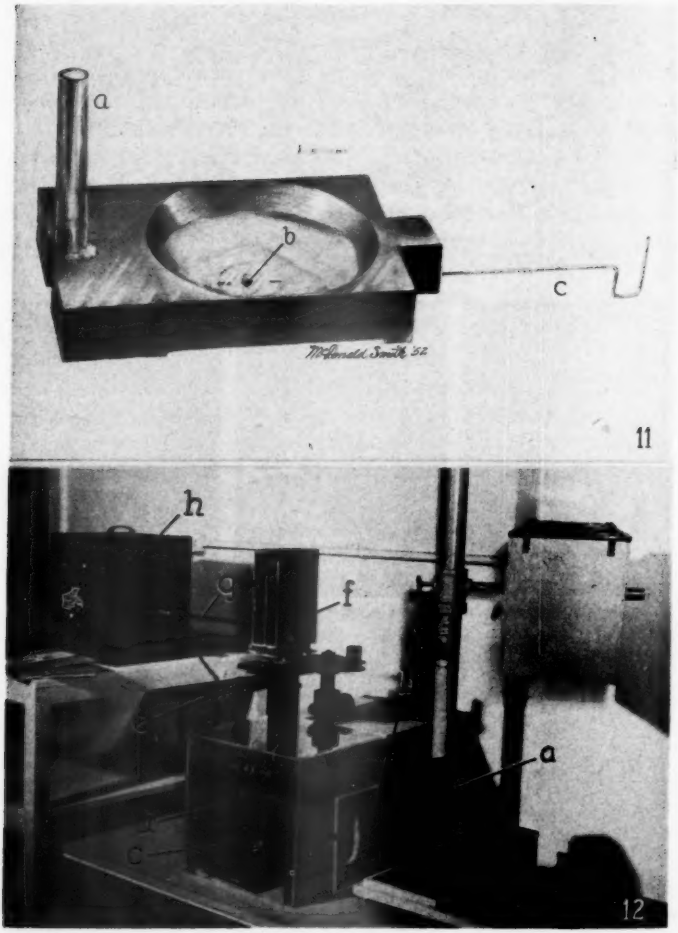

PLATE II

FIG. 11. Perfusion chamber used to observe the effect of changes in environmental conditions on cells in tissue culture. Perfusate, which is introduced into the inflow tube (a) moves across the area occupied by the culture preparation (b) to the outflow tube (c).

FIG. 12. Moving picture set-up for time-lapse, phase contrast cinematography. Light from a ribbon filament lamp (a) passes through a filter system (b) to a compound microscope (c) within a constant temperature chamber (d) maintained at 37° C. The preparation is observed by means of a periscope (e) below a Cine Kodak camera (f) which is actuated via a connecting rod (g) by a time-lapse device (h) (Rolab Co.).

12). Phase contrast optics are employed to make possible observations on the changes in cell structure produced immediately after the introduction of fixatives. Typical features of a cell in a perfusion chamber before (Fig. 13) and immediately after (Fig. 14) the introduction of neutral 10% formalin confirm the suspicion that even under the ideal conditions of rapid fixation, ectoplasmic membrane retraction yields a distorted picture of what in the living state represents dynamic structures. The coagulated nubbins indicated by the arrows in figure 14 correspond to the extremely thin ruffle-like cytoplasmic extensions of the living cell (arrows in Fig. 13).

"Bubble" formation, swelling, shrinking and other phenomena as described recently by Crawford and Barer (1951), who studied the effect of formalin fixation and snail amebocytes and salamander spermatogonia by phase contrast microscopy, are also encountered in our work with cells from mammalian nervous tissue. Neurons and neuroglial cells of man and the laboratory animals are being examined before and after the use of standard fixatives. Data obtained thus far suggests that it may be possible to "re-read" classical textbook pictures of nervous tissues in the light of these results. By way of illustration, imaginative reconstructions have been made from some of Cajal's and Rio-Hortega's well-known drawings to indicate what may have been the appearance of the cells in the living state (Figs. 24, 30, 32). This impertinence may be forgiven by some as a plea in behalf of a more dynamic approach to neurocytology!

Classical illustrations of astrocytes, particularly those of the protoplasmic type, allow for the possibility that the margins of the processes are produced into complex vellum-like membranes. Accelerated activity as seen in cinematographic records of these cells in the living state demonstrates the phenomenon in a dramatic way (Figs. 15–18). The interlacing of processes bearing long ribbon-like undulating membranes produces movements which appear to be somewhat spiral in type resulting in a turbulent effect in the medium resembling intersecting ripples on the surface of water. This was especially well seen in the activities of astrocytic elements which were cultivated from explants of the spinal cord of an adult cat. Typical frames

are represented from these film sequences in figures 19 and 20. However, more satisfactory pictorial representations of this group of cells are shown in figures 21 and 22. Utilizing one of Rio-Hortega's (1949) drawings of a glial cell (Fig. 23) we made a reconstruction of its possible appearance in the living condition (Fig. 24). The irregular "shreds" usually represented in textbook illustrations undoubtedly are the result of the coagulation of delicate cytoplasmic ruffles. Should it become possible to prove that the cytoplasmic activities of astrocytes as seen *in vitro* actually obtain *in vivo*, a powerful mechanism would appear to be available for the movement of metabolites in the intercellular areas of nervous tissue. Studies in progress with the use of particulate substances are attempting to determine if directional pathways for the distribution of fluid are developed *in vitro*, the rate of circulatory flow and, if possible, the force exerted by this system of propulsion. Information regarding the amorphous ground substance of nervous tissue is urgently needed in relation to this thesis.

Astrocytes from the spinal cord of the adult cat have been observed to show marked changes in the appearance of their vacuoles. In the course of cinematographic records, they have been seen to disappear with considerable rapidity but not as rapidly as those of protozoan contractile vacuoles (Figs. 25–28). This phenomenon in glia may be related to some special nurse-like nutritive role in their relation to neurons.

The arrangement of astrocytes *in vivo* as determined by their association to blood vessels via foot processes which constitute one pole, and their free prolongations in other directions, begs a study of patterns of movement over their entire surface. A classical illustration (Fig. 29) from Cajal (1913) has been redrawn

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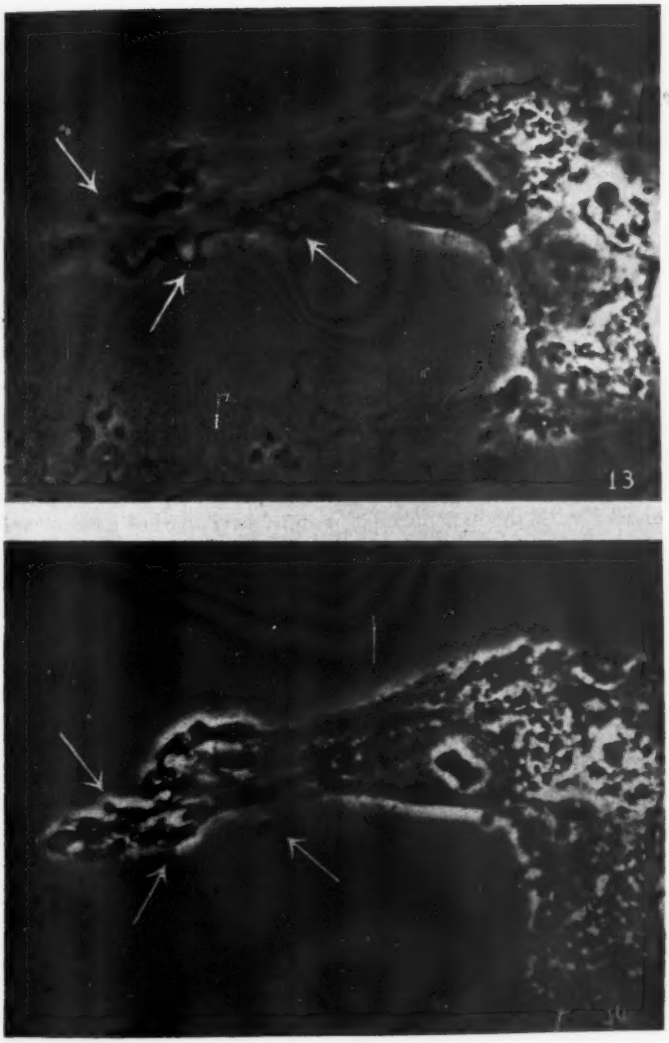
PLATE III

FIGS. 13 AND 14. The effect of fixation as seen with the use of a living cell of the mesenchymatous type in a perfusion chamber and studied with phase microscopy.

FIG. 13. Appearance of the cell immediately before and, in figure 14, after the introduction of neutral 10% formalin. Arrows have been used to indicate changes in thin cytoplasmic ruffles of the type showing marked activity in cinematographic records. Treatment of such material with metallic deposits may further alter the appearance of what existed in the living cell.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(Fig. 30) in an effort to visualize the possible appearance of foot processes in the living state (see also Figs. 31 and 32). Again we have used the argument that string-like processes and "nubbins" in fixed material containing metallic deposits can be legitimately "expanded" in a reverse direction as suggested by our observations on the effect of fixatives. Our drawing leads to the conclusion that vessels of certain categories may be almost completely enveloped by membranous foot processes. Cinematographic records have given some evidence that contractions of a slow type may occur along the course of long astrocytic processes. By virtue of their perivascular feet, tensions might be exerted along such prolongations which could influence vessels of particular importance in brain nutrition. It is conceivable that if such movements led to damming up blood flow, an increase in vascular permeability might result. The significance of these suggestions in relation to the concept of the blood brain barrier may deserve much further study.

The enormously important task of moving metabolites, together with the exigencies of a rapid exchange of gaseous substances, requires a highly efficient "go-between" system of cells lying in the tissue spaces separating blood vessels and the neurons. Given this vital demand for effective transport, which is probably met by a highly organized complex of glial elements, perhaps it is here that one should look for the inability of nerve cells to achieve regenerative function within the CNS. Any interruption in the pattern of supply lines, together with the barrier of gliosis erected to meet structural weaknesses created by injury, would be certain to interfere with the efficacious flow of metabolites essential for continued regenerative function of neurons. While the answer rests unquestionably in a multiplicity of factors, interruption in the dynamic role which the glia normally subserve is very probably involved in this phe-

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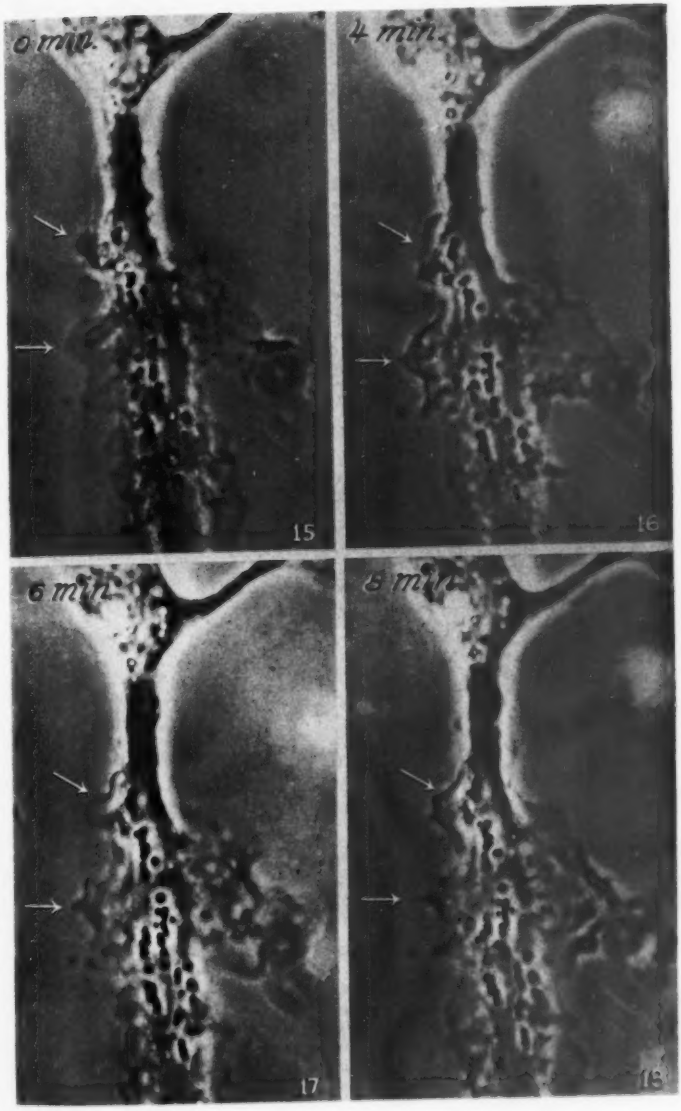
PLATE IV

FIGS. 15–18. Changes in the form of membranous ruffles along the course of a process of what was believed to be an astrocyte from a 10-day culture of the cerebellum of a newborn cat. Arrows have been used to call attention to the appearance of particular areas at different time intervals. Interval pictures taken with a dark phase 1.9 mm. objective (AO) in an ordinary photomicrographic set-up.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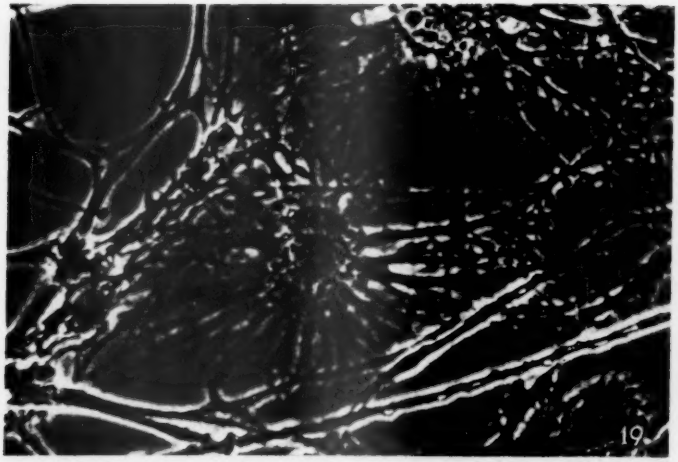

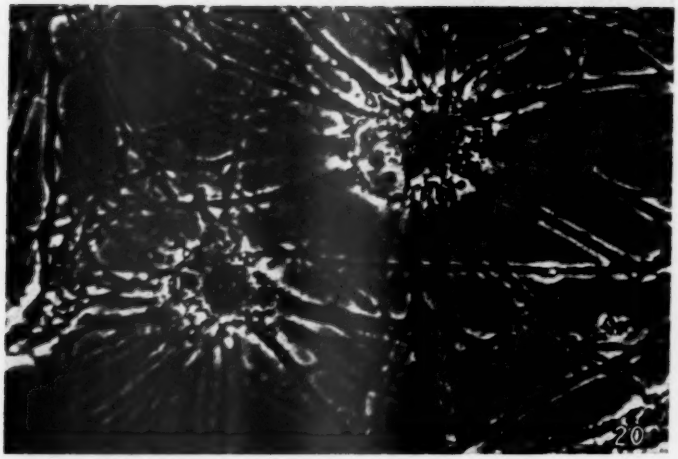

PLATE V

FIGS. 19 AND 20. Enlargements from two frames of a 16 mm. cinematographic record separated by five hours in time, showing two astrocytes in a 22-day culture from the spinal cord of an adult cat. In the projection of this sequence, which was taken at one frame per minute with a dark phase oil immersion objective, the radiating processes showed marked membrane activity of the type indicated by figures 15–18 which resulted in a ripple-like configuration.

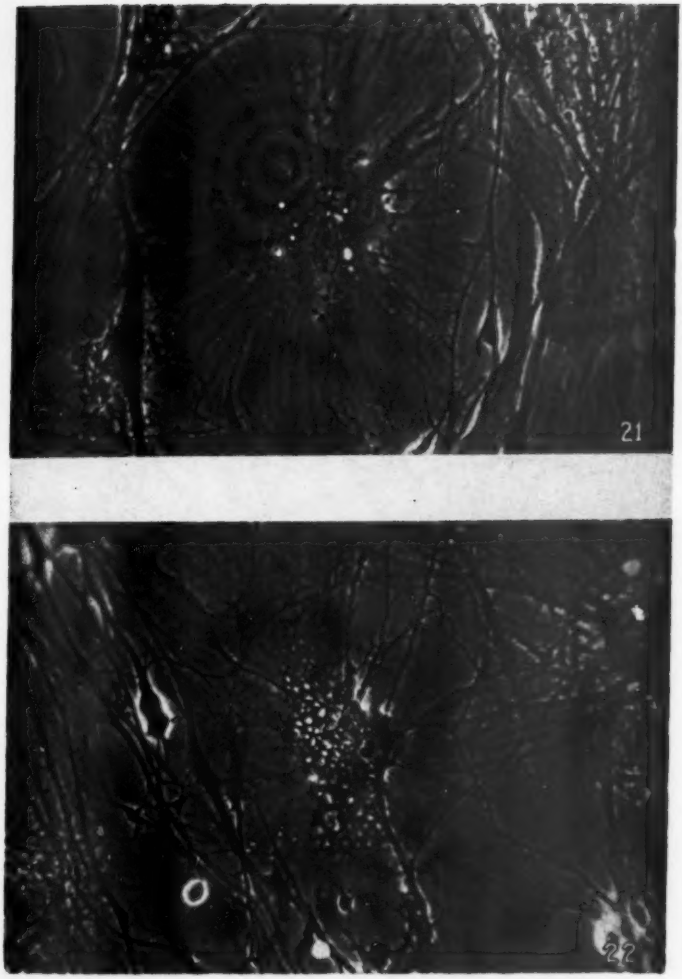

PLATE VI

FIGS. 21 AND 22. Living cells of the astrocytic type from a 22-day culture of the spinal cord of an adult cat as seen in direct photographs which reveal details of structure more faithfully than the enlargements of 16 mm. film strips shown in figures 19 and 20. Considerable variation is encountered in the amount of cytoplasm and the degree of dissection of the processes. Moreover, injury of the type which results from prolonged exposure to microscope illumination leads to progressive dissection of processes and shrinking of membranes resembling the fixation artifact.

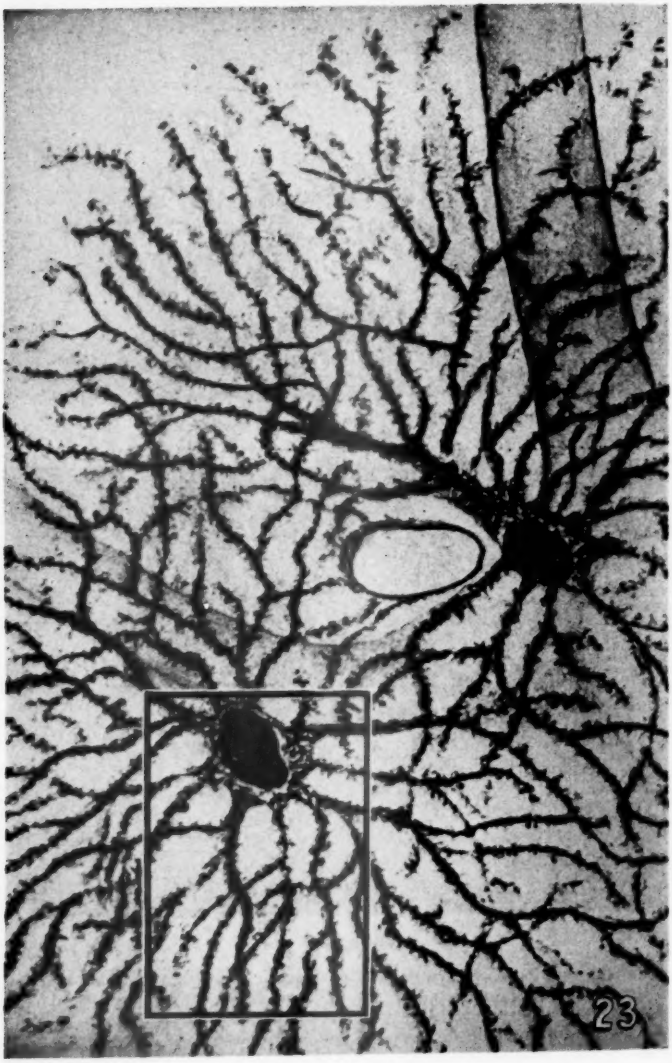
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PLATE VII

FIGS. 23 AND 24. A reconstruction of the probable appearance of a living astrocyte from a classical drawing derived from fixed metallic preparations. The portion indicated by the inset in figure 23, taken from Rio-Hortega's (1949) drawing, was reconstructed in figure 24 on the basis of the study of living glia as shown in figures 15–22 and with the direct evidence of fixation injury effects revealed in studies illustrated by figures 13 and 14.

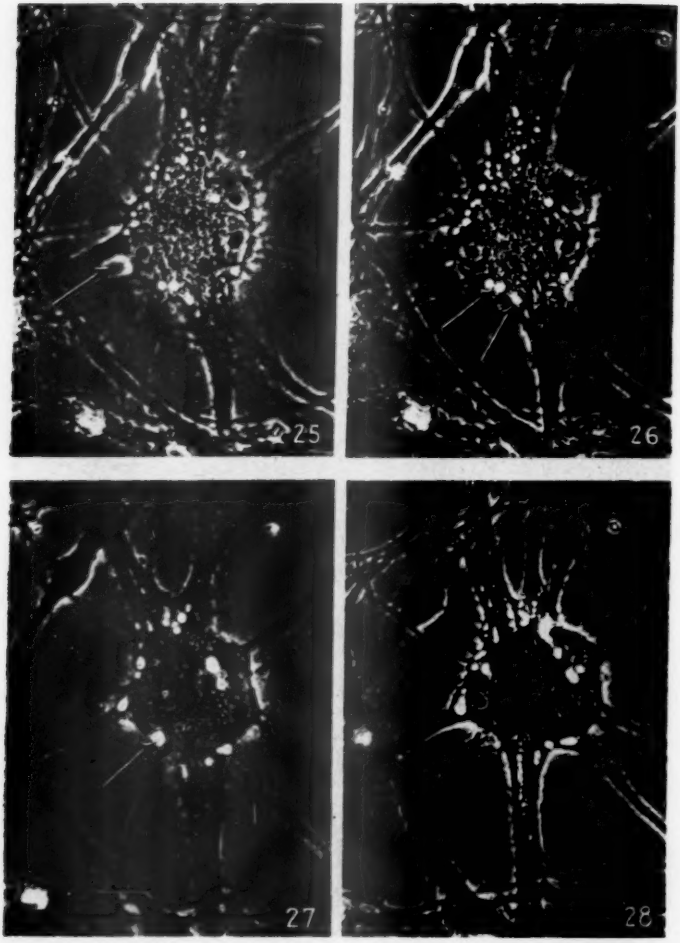
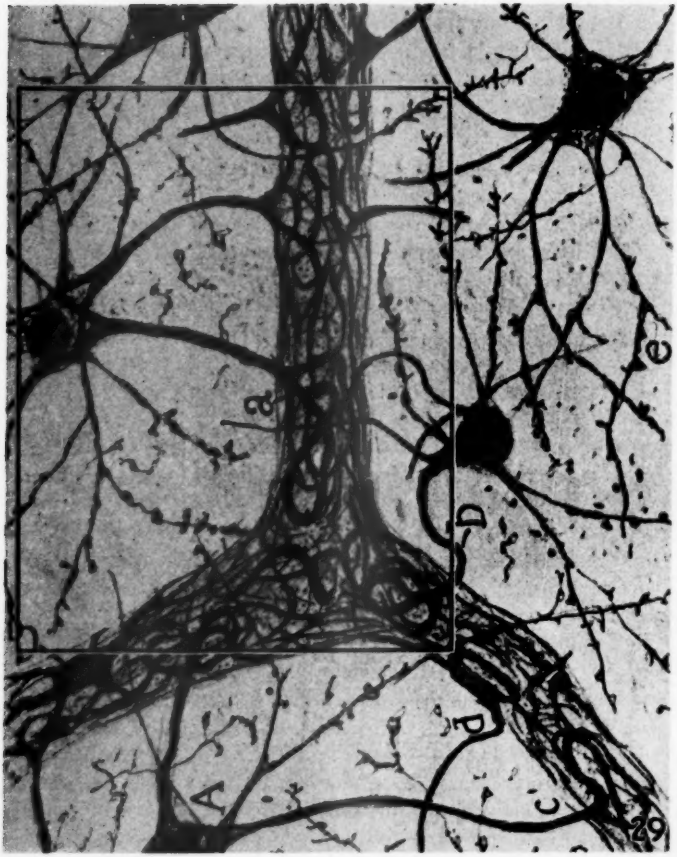


PLATE VIII

FIGS. 25–28. A trinuclear cell of astrocytic type which showed a tendency to the rapid elimination of vacuoles as revealed by time-lapse cinematography. The vacuole indicated by the arrow in figure 25 had almost disappeared 36 minutes later (Fig. 26). Arrows also have been used in figures 26 and 27 to call attention to other vacuoles which showed similar behavior. Vacuolar formation appeared to be related to membrane movement, particularly to pinocytosis (cell drinking), suggestive of high metabolic activity.

nomenon. That neurons can effect some degree of regeneration is evidenced, for example, by the neuroma-like structures produced following the severing of processes by extravasated blood resulting from rheumatic fever, as described by Costero (1949).

In terms of views advanced here concerning the functional role of glia, the report by Costero and Pomerat (1951) that neurons from the adult human brain showed an extraordinary survival in tissue culture can be explained on the basis that nerve cells, freed from the limitations imposed by crowded cellular populations existing *in situ* and faced with the "open" conditions of life in a plasma clot where gas diffusion and chemical exchanges are readily accomplished, can become established providing a minimum of injury has been incurred during the process of preparing the explant. In tissue culture the baggage of specialized differentiations and organells associated with impulse transmissal by neurons is unloaded in favor of demands for adjustment to the new life situation. Neurofibrils and myelin are promptly lost, axonic differentiation is reduced or abandoned and Nissl substance is immediately utilized. There is evidence that under favorable conditions these characteristic features of the neuron are eventually recovered (*cf.*, Lumsden, 1951). Peterson (1950) demonstrated the genesis of myelin *in vitro* in nerves from the ganglia of chick embryos as a progressive activity. The development of dendritic and axonic processes in tissue culture outgrowths has been illustrated for the Purkinje cell of human fetuses and newborn individuals maintained for more than three months *in vitro* (Hogue, 1950). The persistence of neurofibrillar differentiation, at least in cells of embryonic origin, is well-known to workers who employ the tissue culture method for the study of nervous tissue. Meyer and Jablonski (1937) were able to maintain these structures in cultures of chick embryo ganglia for 19 weeks. While Nissl substance and neurofibrils were not clearly demonstrated, Murray and Stout (1947) were able to show that neurons from adult human ganglia not only maintained polarity and produced neurites approximately 2 mm. in length after two weeks *in vitro*, but such cells actually exhibited the capacity to undergo mitosis. The re-formation of Nissl substance, neurofibrils and



axonic differentiation of adult neurons from the human brain *in vitro* is under study in our laboratory.

If what has been described of astrocytic activity concerns the interstitial (interneural) or matrix area, the pulsatile movements of oligodendroglia and related elements involve parenchymatous (intraneural) or neuroplasmic dynamics. In the closing years of his life Rio-Hortega, the discoverer of the oligodendroglia, believed that these cells formed a part of a family of

elements closely applied to the perikaryon and axonic processes which might include not only satellite cells and the interfascicular elements associated with myelin in the brain but also Schwannoid cells in the spinal cord, amphicytes in ganglia and perhaps even the Schwann cells of peripheral nerves.

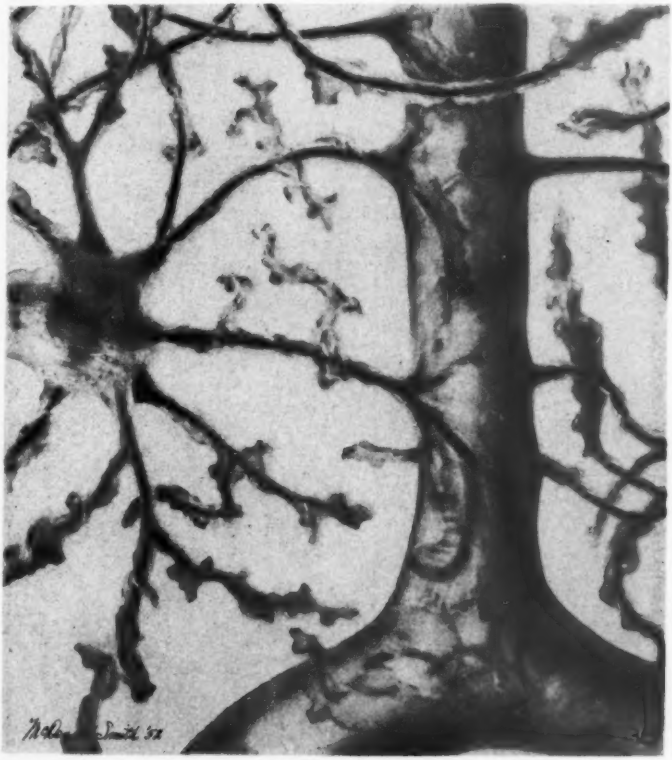
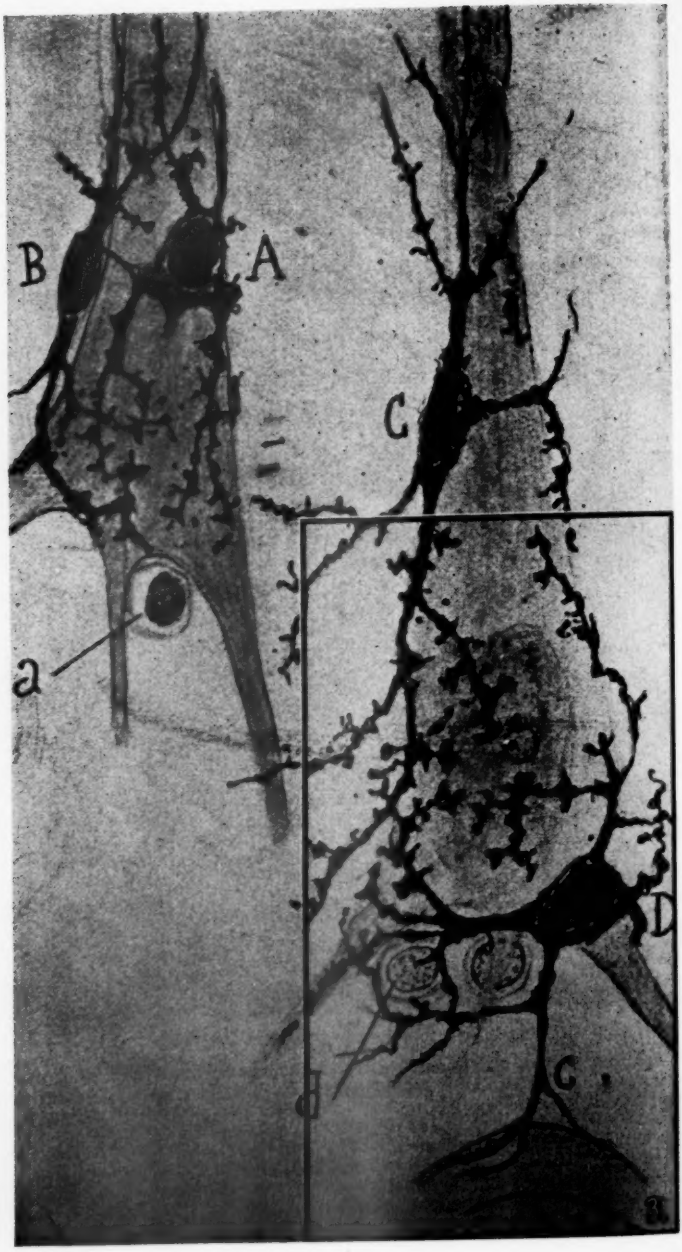


PLATE IX

FIGS. 29 AND 30. An attempt at reconstructing the appearance of living astrocytes, particularly of their foot processes. A classical metallic preparation drawn by Cajal (1913) is shown in figure 29 and with the premises given in the description of figures 23 and 24, the probable appearance of various structures within the inset is shown in a reconstructed drawing (Fig. 30). Stringy and strap-like components of the foot processes have been expanded as they might have been in the living state which suggests an almost complete encasement of the blood vessel.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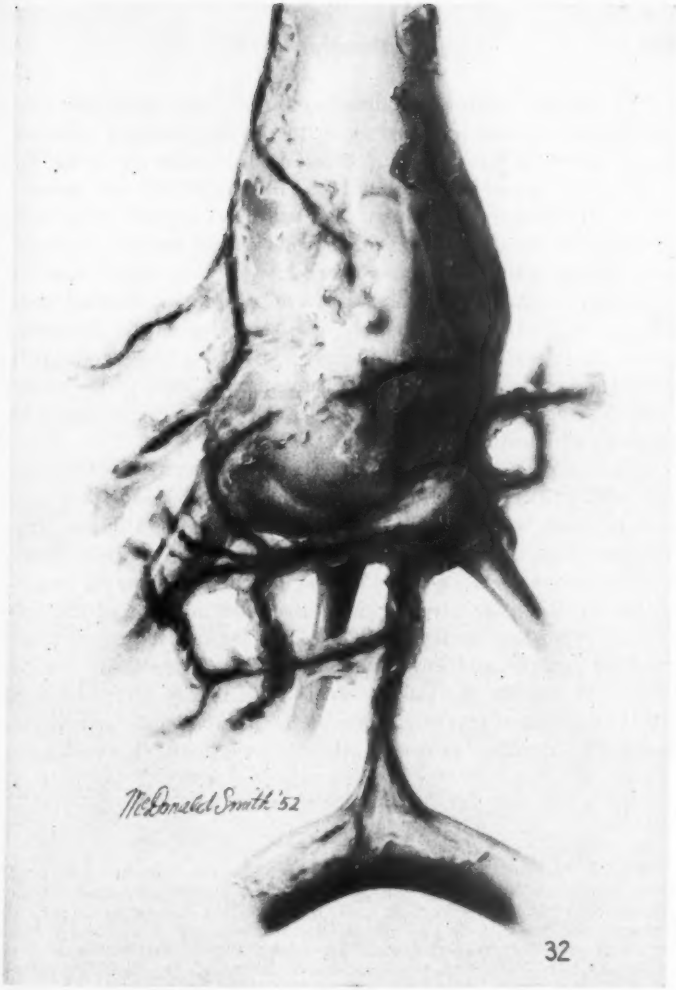

PLATE X

FIGS. 31 AND 32. The area of the inset in a drawing (Fig. 31) by Cajal (1913) has been reconstructed so as to show the probable structure of glial elements intimately associated with the perikaryon of nerve cells as they might appear in the living state (Fig. 32). Twiglets and nubbins typical of fixed "silver-plated" preparations have been redrawn as foliose processes with extremely thin margins. Without exaggerating this technique of interpretation it becomes obvious that the cell bodies of neurons might be held in the extensive clasp of satellite cells. Should such glial elements have an inherent rhythmic contractile activity, a "massaging" action might be exerted on the neuroplasm. The presence of a foot process extending from the glial cell is of special interest to the speculation presented in this thesis.

The finding that oligodendroglia exhibit a slow rhythmic contractile activity has initiated experiments to determine whether this function is characteristic of all members in the series. In seeking the possible functional role of such cells in nervous tissue, attention was obviously directed to the association of their processes to the perikaryon of neurons and to axonic prolongations. Here again it becomes necessary to re-interpret classical neurohistological preparations in terms of what is learned with the use of tissue culture techniques. While generally filamentous at their point of origin, processes of living oligodendroglial cells frequently show blade-like expansions with considerable ruffle-like activity. These have been illustrated by photographs made by film sequences (Pomerat, 1951a).

Satellite cells of the type shown in the drawing (Fig. 31) by Cajal (1913) actually may have had membranous processes which could "clasp" a relatively large surface area of the perikaryon (Fig. 32). Under such conditions contractile activity might serve to exert a gentle "massaging" action upon the neuroplasm. In his exquisite drawings of oligodendroglia of the fascicular type, Rio-Hortega (1921, 1928) represented their processes as strands and bands having varying degrees of density (Fig. 33). On the basis of observation on living cells, it is very likely that these processes form delicate envelopes around the myelin as complete as the sheath of Schwann but that, as in the

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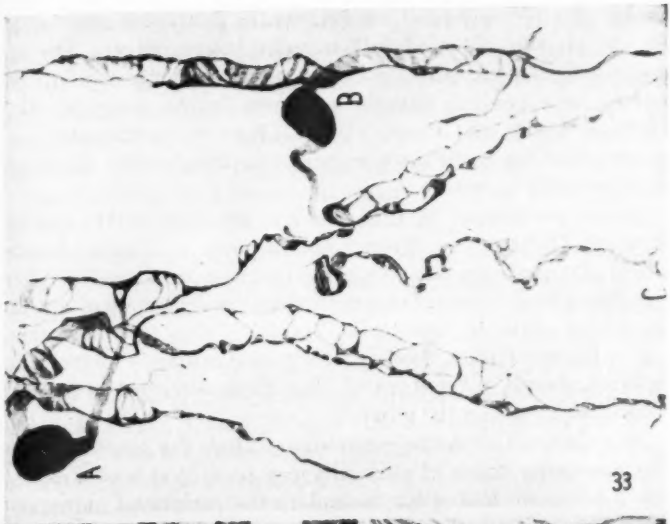
PLATE XI

FIG. 33. Vellum-like cytoplasmic processes of oligodendroglia associated with myelinated nerves are represented in this drawing by Rio-Hortega. In the light of the argument that fixation used for neurohistological preparations damages delicate expansions of cytoplasm, it is possible that in living tissue nerve fibers are surrounded by a continuous envelope.

FIG. 34. An illustration of an autonomic ganglion reproduced from Rio-Hortega, Polak, and Prado (1942) suggesting the extent to which glia may clasp both the perikaryon and the processes of neurons. In their article the authors concluded that "The so-called 'capsular cells' are *perisomatic* gliocytes, polygonal, laminar or stellated in shape, which spread on the neuronal surface or envelop it in a plexus of slender processes. The so-called 'subcapsular cells' are commonly periaxonic gliocytes, mono, bi, pluripolar or laminar in shape, which wrap the nerve fibres in their glomerular or pericapsular parts. There are transitions between them and the Schwann cells."

"Histo-physiologically considered, the perisomatic elements are equivalent to cerebral astrocytes, and the periaxonic ones to oligodendrocytes of the central third and fourth types."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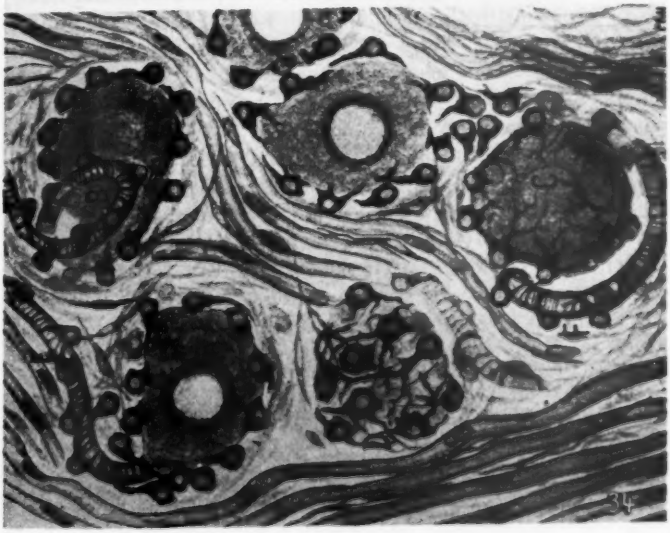
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case of the microglial cell, coagulation by fixation is responsible for the pictures obtained with metallic impregnations. The sequence of elements enveloping the perikaryon and myelinated nerves in autonomic ganglia as shown in the study by Rio-Hortega, Polak and Prado (1942) (Fig. 34) invites the suggestion that many neurons may rest entirely within the clasp of a pulsatile system!

It was pointed out by Lumsden and Pomerat (1951) and by Pomerat (1951a) that the pulsatile activity of oligodendroglia might play an important role in the metabolism of myelin. After reviewing many years of observation on the deposition of myelin in the tail of the living tadpole, Speidel (1950) concluded that, ". . . In my opinion, there is a reciprocal nutritive relationship between sheath cell and nerve fiber. Each one exerts a marked trophic influence on the other."

Speculation also can be extended to include the possibility that the massaging action of glial cells may provide at least a part of the mechanism looked for to explain the peripheral movement of substances with the axoplasm. Reviewing this problem, Gerard (1950) states, "More recently, direct evidence of the peripheral movement of material of the axon has come from several sources. Weiss *et al.* (1945) showed rapid transport of tracer ions by endoneurial fluid, and Weiss (1944a) also found a slower transport of axoplasmic contents, piled up behind a temporary constriction. Young (1945) likewise obtained direct histological evidence of material flow into axones; and Hyden and his colleagues (Hyden, 1943; Hamberger and Hyden, 1949; Hyden and Hartelius, 1948) inferred, from changes in the ultra-violet absorptions spectrum under conditions of activity, chromatolysis, etc., that nucleoprotein moves from soma into processes of the neuron. But the movement is actually more af a mystery than ever."

Coming to grips with this "mysterious movement" involved in the nerve growth, Weiss (1950) has written, "The motive force of the elongation is not quite understood. While Lewis ascribes it generally to the contractility of the superficial gel layer, it may become necessary to assume a rhythmic alternation between contractility and relaxation residing in the axon surface and moving the axoplasm forward in the manner of a peristaltic

wave. The neuron seems to possess a peculiar pumping mechanism by which material from the cell body can be forced distad all the way to the tip; evidently, this is the same mechanism which, after the fiber has ceased to elongate, continues to pump in axoplasm, which then accrues to the width of the fiber. At any rate, the elongation of the nerve process is essentially a phenomenon of protoplasmic motion rather than of true growth."

". . . The nerve tip is drawn out along such linear threads as if by capillarity. The actual cause of this adhesivity is not known, but it does not seem to reside in simple surface tension and wetting properties, or at least not exclusively so. It exerts a certain pull at the tip of the fiber, thus giving direction to the push of the pumping mechanism dsicussed before. Because of this dual action, I have termed this a 'pull-push mechanism' (Weiss, 1944b). Guidance by fibrous interfaces is an active principle and not a passive one, such as 'least resistance' (Weiss, 1941)."

Beyond the problems of axonic outgrowth and nutrition, as well as of the metabolism of myelin, the association of oligodendroglia with small vessels invites further study with living preparations.

Current success in the cultivation of both neurons and glia with modern methods of tissue culture, together with the advantage of using phase contrast microscopy in making time-lapse cinematographic records of cell movements, provides an important challenge for the re-examination of old problems not only concerning the nutrition of the brain and of regenerative phenomena but, perhaps, the basic aspects of impulse conduction. Electronic devices for recording disturbances flowing across single nerve fibers emigrating in tissue culture can also be utilized for recording action currents from individual neurons, oligodendroglia or groups of pulsatile glial cells. It is possible that such studies might assist in elucidating the nature of electroencephalographic disturbances in addition to clarifying fundamental problems of intracerebral tissue fluid movement.

The use of the perfusion chamber, which permits recording changes in cellular behavior following the introduction of chemical variables under highly controlled conditions (Pomerat,

1951b), adds fresh opportunities for methodological neurophysiological studies. Thus the mobility manifestations of undulating membranes, mitochondria, granules and other structures can be recorded from various species of neurons and glial cells derived from normal and pathologic human tissue. With the use of such data to serve as a base line, the effect of various physical and chemical factors, including clinically important drugs, on particular cell types can be explored. The action of CNS stimulants, depressants, anesthetics and anticonvulsants upon the rhythmic pulsatile activity of the oligodendroglia, for example, might become of great importance in evaluating the place of this most common cellular component of the family of brain cells in cerebral physiology.

In very loose discussions regarding the "nurse" function of glia in assisting neurons with the solution of their complex metabolic problems, I have found it useful to draw an analogy between the problem of an enormous human population living in the comparatively small area of Manhattan Island and the conditions which face millions of neurons in the relatively limited mass of brain tissue. The efficiency of the energetic citizens of New York is so critically dependent upon subway, bus, truck, train and ship transport that a strike by the workmen in any of these agencies produces near panic in human activity within relatively few hours. Strikes by elevator operators are forbidden in New York's democracy because these have been known to result in the death of the occupants in tall buildings. Efficient neuronal performance is also critically dependent upon an exquisitely balanced series of transport devices. High energy requirements and the necessity for the rapid removal of waste in situations where neurons cannot rest directly upon capillary beds may require specialized mechanisms provided by astrocytes for interstitial fluid movement. The dependence of the neuroplasm, even at the tips of axonic processes at relatively enormous distances from the perikaryon, upon nuclear or perinuclear activity may demand an intracellular circulatory device of the very particular type suggested by the pulsatile oligodendroglial cell. It is important to emphasize that while we have been made aware of fluid movements in tissue compartments in other parts of the body, the dynamics of "juices" in nervous

tissue have received far too little attention. Vague statements regarding the production of headaches by intracranial fluid pressure and the removal of water by aspirin actually may prove to be very useful leads for further inquiry!

And so we see that neurological investigation remains what was described by Cajal as ". . . a delicious rapture, an irresistible enchantment." After having known the thrill of seeing extraordinary movement in cells from nervous tissue as revealed by motion picture films, you can imagine my joy in coming across a passage in Cajal's "Recollections of My Life" (1937) which showed him not only to be prophetic and imaginative but thoroughly modern in his dynamic approach to morphology:

". . . the garden of neurology holds out to the investigator captivating spectacles and incomparable artistic emotions. In it, my aesthetic instincts found full satisfaction at last. Like the entomologist in pursuit of brightly coloured butterflies, my attention hunted, in the flower garden of the gray matter, cells with delicate and elegant forms, the mysterious butterflies of the soul, the beating of whose wings may some day—who knows?—clarify the secret of mental life."

Acknowledgment

Grateful acknowledgment is made to Mr. George Lefeber and to Mr. Curtis Hoskins for indefatigable effort in recording the characteristics of living cells with phase photomicroscopy and to Mr. McDonald Smith for painstaking studies in the reconstruction of classical illustration of glial cells in terms of their probable "living" appearance.

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FREQUENCY OF CANCER IN THE WHITE AND COLORED RACES AS OBSERVED AT AUTOPSY BETWEEN 1920 AND 1949 AT THE MEDICAL BRANCH

R. H. Rigdon, Helen Kirchoff, and Mary Lee Walker*

The problem of the frequency of cancer in the white and colored races was studied by Pearl and Bacon (1927) in the autopsied cases at the Johns Hopkins Hospital. They observed that "in this necropsied population, malignant tumors of all sorts taken together occur from two to three times more frequently among white persons than they do among colored persons." Holmes (1935) studied this same problem using data obtained from Vital Statistics, and found that "in the data for total cancer it is apparent that the differences in the mortality of white and colored males are not great in the earlier age groups and that they increase rather rapidly in the more advanced ages, becoming over twice as high for white males in ages over sixty-five." In reviewing the deaths and death rates for total cancer in Alabama, Georgia, Mississippi, Louisiana, Virginia, and North and South Carolina, Holmes found "the cancer death rates in the younger age groups are higher in the colored population than in the whites, while the reverse relation occurs in the older ages."

The cases autopsied at the Medical Branch Hospitals of The University of Texas over a period of thirty years have been reviewed to determine the frequency of cancer in the white and colored races (Table 1). For this study the stillborns and cases less than twenty years of age are not included. Five thousand two hundred and sixty-four autopsies were performed on individuals twenty years of age and older from 1920 to 1949. In this group of consecutive autopsies there were 929 cases with a malignant tumor, an incidence of 17.6 per cent. Usually death resulted from the tumor; however, in a few cases the cancer was an incidental finding at autopsy. The colored race included only Negroes, while all other races were included as whites.

Graph I shows the distribution according to race and sex of the 5,264 autopsies on individuals twenty years of age and older.

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*From the Department of Pathology, The University of Texas Medical Branch, Galveston, Texas. Received for publication July 2, 1952.

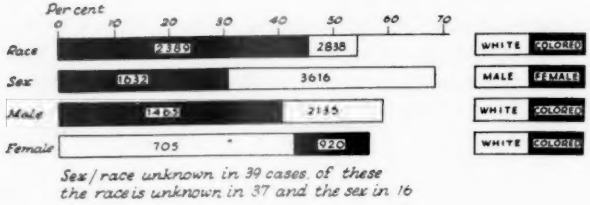
It may be seen in this graph that the percentage of whites autopsied is 53.9 and the percentage of colored, 45.4. The percentage of autopsies on males is 68.7 and on females, 31.0. Of the males, 59.3 per cent are on the whites and 40.7 per cent on the colored; 56.6 per cent of the females are colored and 43.3 per cent, white.

TABLE 1

Number and age of cases autopsied between 1920 and 1949

Year	Age Group													
	Still-born	Under 1	1–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	90+	Age Unknown	Total
1920....	0	3	3	7	10	17	7	8	10	2	0	0	27	94
1921....	0	6	1	1	8	14	17	13	7	2	1	0	23	93
1922....	0	5	2	1	15	22	18	16	10	9	3	0	5	106
1923....	0	5	0	2	12	16	21	13	12	6	3	0	3	93
1924....	0	5	4	2	9	10	15	17	8	6	1	0	11	88
1925....	0	7	1	3	20	27	16	16	13	5	5	0	12	125
1926....	4	9	6	4	18	24	32	15	17	9	2	0	4	144
1927....	3	11	6	6	23	35	17	20	19	9	6	0	0	155
1928....	2	10	1	2	17	23	31	26	11	14	0	0	0	137
1929....	7	3	6	6	20	26	36	25	23	9	4	0	2	167
1930....	14	9	7	6	18	22	28	30	18	11	1	0	1	165
1931....	7	16	7	9	14	29	44	34	24	9	3	0	1	197
1932....	4	7	5	7	28	35	62	37	21	11	1	0	0	218
1933....	9	10	13	5	19	29	47	45	40	16	3	1	1	238
1934....	16	23	14	7	23	27	50	45	46	18	8	0	1	278
1935....	16	16	9	13	30	53	62	50	40	23	5	1	2	320
1936....	11	27	11	7	32	35	54	56	42	17	5	1	3	301
1937....	6	18	12	7	23	37	48	39	27	22	2	1	5	247
1938....	6	28	7	4	22	23	38	37	35	19	3	0	4	226
1939....	6	26	12	10	22	27	32	59	43	32	3	0	3	275
1940....	14	23	16	6	22	37	42	52	48	29	3	1	0	293
1941....	8	32	16	8	14	24	28	45	33	27	9	0	0	244
1942....	8	39	14	12	25	29	29	49	36	19	4	0	4	268
1943....	13	33	12	17	24	31	21	37	43	24	5	1	7	268
1944....	3	52	17	8	20	21	25	36	37	17	6	0	10	252
1945....	4	33	16	20	18	27	27	38	28	19	8	2	15	255
1946....	17	45	10	9	27	22	44	68	48	31	3	2	3	329
1947....	19	52	14	13	22	43	64	63	62	31	12	3	0	398
1948....	8	50	19	12	17	21	53	65	54	28	5	3	0	335
1949....	4	45	26	17	15	27	57	52	46	31	8	0	2	330
TOTAL	209	648	287	231	587	813	1,065	1,106	901	505	122	16	149	6,639

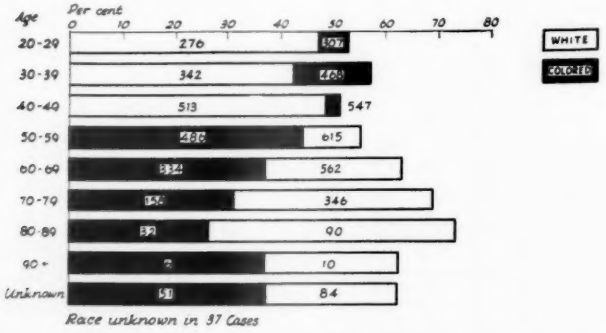
The age and race of the autopsied cases are shown in graph II. The number of colored autopsies is greater than the number of white in all groups less than fifty years of age, but the number of autopsies on white individuals is greater in the groups fifty years of age and older.



GRAPH I

The race and sex of 5,264 individuals 20 years of age and older studied at autopsy.

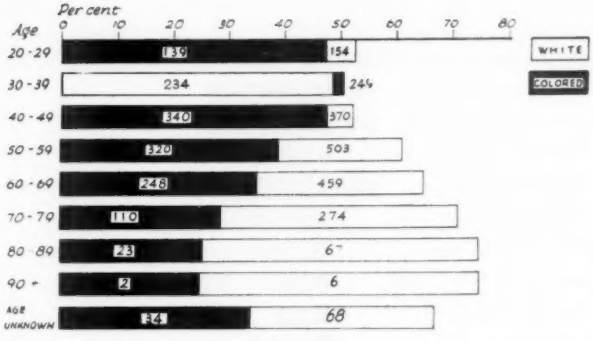
The ratio of white males to colored males in the various age groups in the 3,600 male autopsies is given in graph III. The ratio of white to colored males is small between twenty and forty-nine years of age; however, after fifty years of age this ratio becomes progressively greater. The number of colored females exceeds the number of white females in each age group between twenty and fifty-nine years, while this ratio is reversed at sixty and above (graph IV).



GRAPH II

The percentage of cases of the two races in the various age groups in 5,264 autopsy cases.

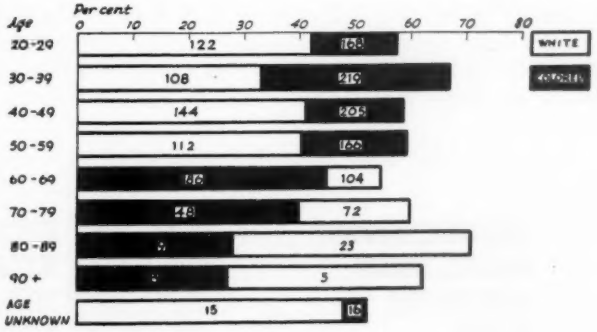
In the group of 5,264 autopsies there were 929 individuals with malignant tumors. The primary sites for these are shown in graph V. In forty-four of these cases the primary site of



GRAPH III

The percentage of white and colored males in various age groups in the 3,600 male autopsies.

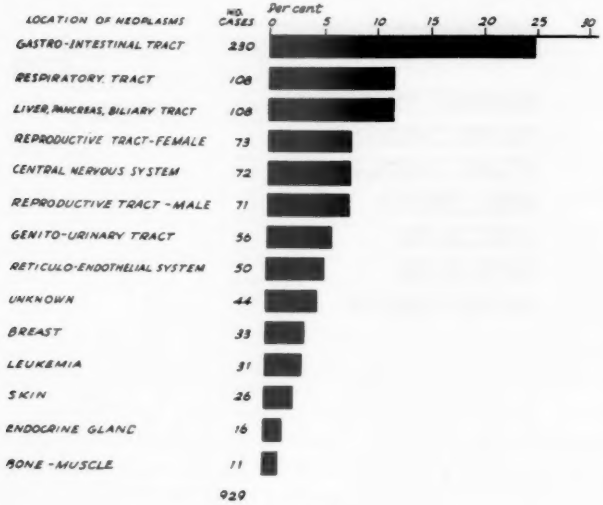
origin was undetermined. Brain tumors, leukemia and tumors of the reticulo-endothelial system are included in this study. The gastro-intestinal tract is the most frequent site and neoplasms of the respiratory tract and of the liver, pancreas and biliary tract are second.



GRAPH IV

The percentage of white and colored females in the various age groups in the 1,625 female autopsies.

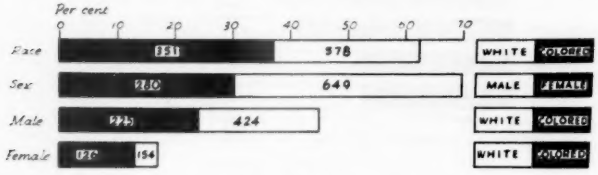
Graph VI shows the distribution of the 929 cases of cancer according to race and sex: 62.2 per cent of these cases occur in the white race and 37.8 per cent in the colored. The ratio of males



GRAPH V

Primary site of the 929 neoplasms as observed at autopsy.

with cancer to females with cancer is 69.9 to 30.1 per cent. There are approximately twice as many cases with neoplasms in the white males as in the colored males, while the number of cases in the white females only slightly exceeds that in the colored females.

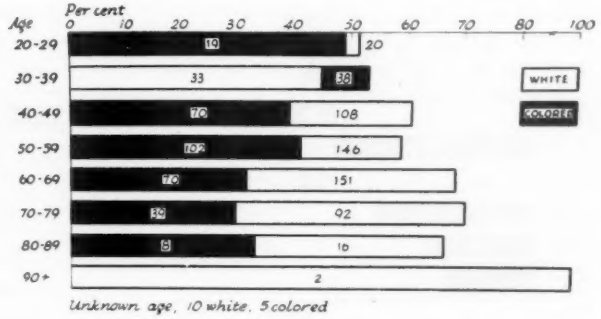


GRAPH VI

The race and sex of 929 cases of malignancy in individuals 20 years of age and older studied at autopsy.

The ratio between white and colored people with cancer in the different age groups is shown in graph VII. The number of

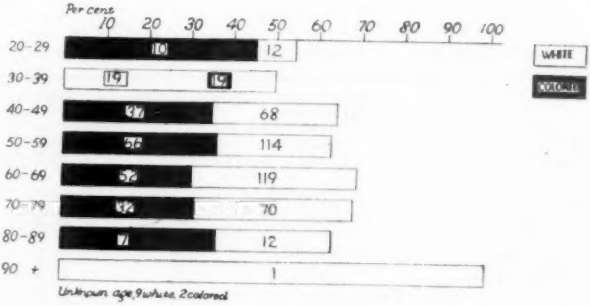
colored exceeds the number of whites only in the age group from thirty to thirty-nine years. After the age of forty the predominance of whites over colored is progressively increased.



GRAPH VII

Percentage of white and colored cases in the various age groups in 929 autopsies with malignant tumors.

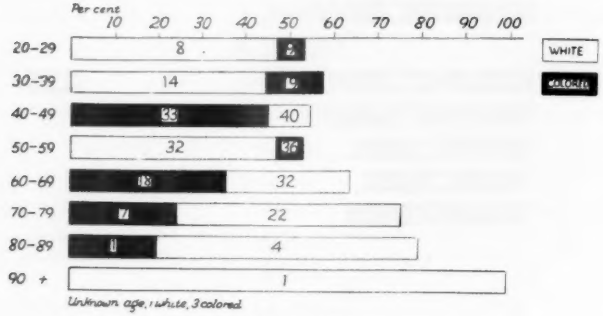
Graph VIII is an anlaysis of the 649 autopsies of males with malignant tumors. The number of white males exceeds the number of colored males in all age groups except between thirty and thirty-nine years. In this group there is the same number of colored and white males. The 280 autopsies in females with



GRAPH VIII

Percentage of white and colored males in various age groups in the 649 autopsies of males with malignant tumors.

cancer are shown in graph IX. The number of colored females exceeds the number of white females in the age group twenty to twenty-nine, thirty to thirty-nine and fifty to fifty-nine years. In all other age groups the whites exceed the colored.



GRAPH IX

Percentage of white and colored females in various age groups in the 280 autopsies of females with malignant tumors.

The relative frequency of malignant neoplasms in the white and colored races and the male and female is shown in table 2. Cancers occur in 20.4 per cent of the whites and in 14.7 per cent of the colored. There is essentially no difference in the frequency in the male and female. In males, the percentage is 19.9

TABLE 2

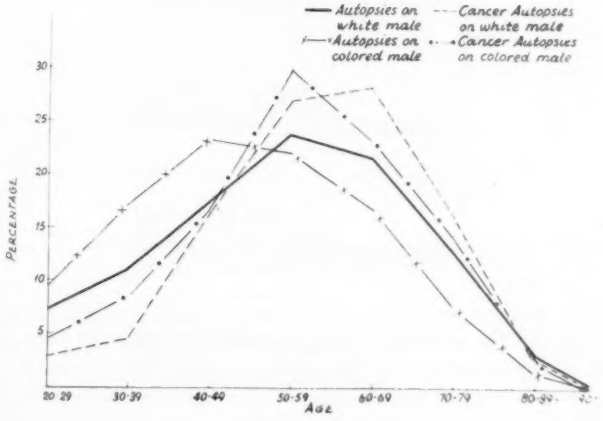
Relative frequency of malignant tumors as studied at autopsy

	Total No. of Autopsies*	Autopsies with Cancer	Relative Frequency
White	2,838	578	20.4%
Colored	2,389	351	14.7
Male	3,600	649	18.2
Female	1,625	280	17.2
White Male	2,135	424	19.9
Colored Male	1,465	225	15.4
White Female	705	154	21.8
Colored Female	920	126	13.7

*Sex and/or race unknown in 39 cases; of these the race is unknown in 37 and the sex in 16.

for whites and 15.4 for colored; in females, the percentage is 21.8 for whites and 13.7 for colored.

The race and age distribution of autopsies on males and of male autopsies with malignant tumors are shown in graph X. The percentage of autopsies in white males is highest between the ages of fifty and fifty-nine, while the highest incidence of cancer in white males occurs between the ages of sixty and sixty-nine. The percentage of autopsies in colored males is highest between the ages of forty and forty-nine, and the highest incidence of cancer in colored males occurs between the ages of fifty and fifty-nine. In the age groups between twenty and forty-nine the relative frequency of colored males is greater than the relative frequency of white males in all autopsies. In the age groups between twenty and fifty-nine the relative frequency of colored males is greater than the relative frequency of white males in autopsies showing malignant tumors.

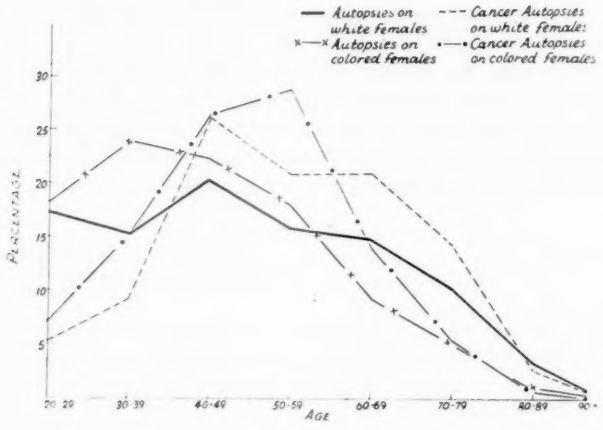


GRAPH X

Age and race distribution in the 3,600 male autopsies and the 649 autopsies of males with malignant tumors.

Graph XI shows the race and age distribution of autopsies on females and of autopsies on females with cancer. The percentage of autopsies in white females is highest between the ages of forty and forty-nine, while the highest incidence of cancer in

white females occurs within the same ages. The percentage of autopsies on colored females is highest between the ages of thirty and thirty-nine, while the highest incidence of cancer in colored females occurs between the ages of fifty and fifty-nine. Between the ages of twenty and fifty-nine the relative percentage of colored females is greater than the relative percentage of white females, both in all autopsies and in autopsies showing cancer.

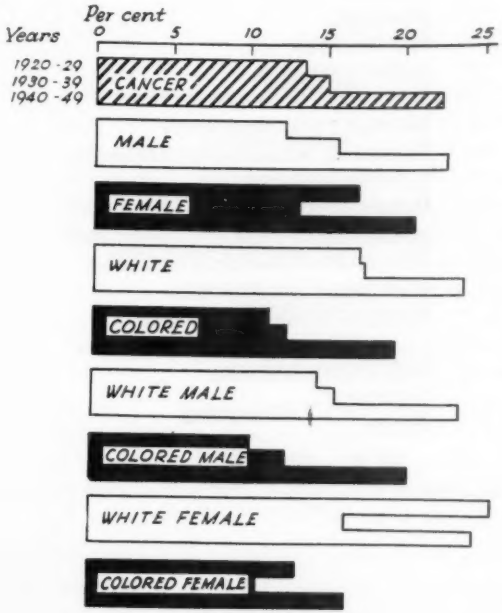


GRAPH XI

Age and race distribution in the 1,625 female autopsies and the 280 autopsies of females with malignant tumors.

The relative frequency of cancer according to race and sex during the three decades from 1920 to 1949 is shown in graph XII. The percentage of all cancers shows a slight increase from the first to the second decade, with a marked increase for the third. Cancer in males shows a progressive increase, with the highest percentage occurring during the third decade. The frequency of cancer in females varies between 1920 and 1949. The lowest percentage occurs during the second decade and the highest during the third. Cancers in the white race show an almost equal percentage during the first two decades, with an increase during the third. In the colored race there is a slight increase in the frequency of cancer from the first to the second

decade and a marked increase in the third. The relative percentage of cancer in the white and colored males shows an increase during the three decades, with the highest percentage appearing in the third. In the white and colored females a fluctuation occurs with the smallest percentage of malignancies appearing during the second decade. The highest percentage in the white females occurs during the first decade and in the colored during the third.

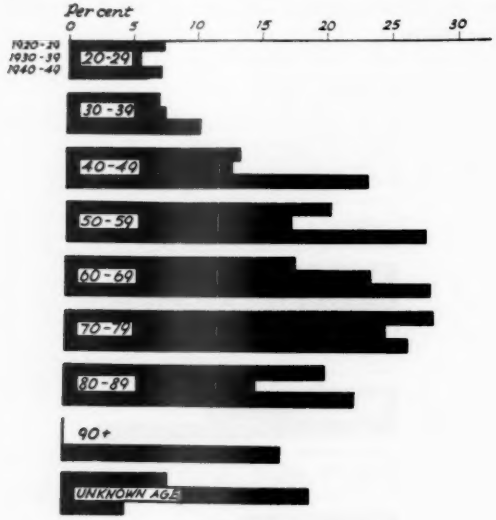


GRAPH XII

Relative frequency of cancer according to race and sex during the 3 decades, 1920–29, 1930–39 and 1940–49.

Graph XIII shows the relative percentage of malignancies in the various age groups during the three decades from 1920 to 1949. The highest percentage appears during the third decade in all age groups except twenty to twenty-nine and seventy to seventy-nine years. In these two groups, the variations are 2

per cent or less between the first and third decade. Only in the age groups thirty to thirty-nine and sixty to sixty-nine is there a progressive increase in the frequency of cancer in all three decades. In all other age groups the frequency is less in the second decade than in the first.



GRAPH XIII

Relative frequency of cancer according to age during the 3 decades, 1920–29, 1930–39 and 1940–49.

The number of autopsies performed, the number of cases with cancer and the percentage of the cases with cancer observed between 1920 and 1949 in both the white and colored male and female are shown in table 3. The percentage of cancer is greater in the white male than it is in the colored male until seventy years of age, at which time the reverse occurs; in the female the frequency is greater at all ages in the white than in the colored.

Discussion

Data obtained from a single hospital and from autopsied cases may not represent a satisfactory sample for statistical purposes;

however, such a study may contribute to our knowledge of neoplasms. In this study of the frequency of malignant tumors in the white and colored races over a period of thirty years, there were 5,264 autopsies on individuals twenty years of age and over, of which 53.9 per cent were in the whites and 45.4 per cent were in the colored. Since no distinction is made in the pathologic studies of the two races, the comparative results would seem to be significant.

There is an increase in the frequency of cancer during 1940 to 1949 when compared with 1920 to 1929 and 1930 to 1939. There is only a slight variation, however, in the frequency of cancer between 1920 to 1929 and 1930 to 1939. The increase in the third decade occurs in all age groups except in those twenty to twenty-nine and seventy to seventy-nine. When considering the frequency of cancer in males and females and in whites and colored during the three decades, sometimes it is either higher or lower in one decade than in the others. The marked increase in the frequency of cancer during the third decade may be attributed to the successful use of therapeutic agents in the treatment of infectious diseases. One would hardly conclude from a study of cancer in these autopsies that all neoplasms suddenly increased in frequency in 1940. This increase in cancer, therefore, is considered to be only apparent and does not indicate an actual increase in the frequency of malignant tumors. A similar opinion relative to the frequency of neoplasms was expressed by Dublin (1945) when he said: "The increase in the proportionate mortality from cancer was due very largely to the continued reduction in the death rate from most other important causes, and in lesser degree to the increasing average age of the policyholder group."

The peak of frequency in the malignant tumors occurs from fifty to fifty-nine years of age in the colored females, ten years later than it does in white females. It occurs from fifty to fifty-nine years of age in the colored males, ten years earlier than it does in the white males. The age of the peak of cancer frequency is the same for the colored males and females; however, in the white males and females there is a twenty-year variation. Pearl and Bacon (1927) found the peak to be from fifty to fifty-nine years for white and colored males, while it was forty to forty-nine for white and colored females. Tomlinson (1945)

found that the peak of frequency in malignant tumors in the British West Indians occurred between fifty-one and sixty years in the male and between forty-one and fifty years in the female; in the Panamanian Negroes it occurred between twenty-one and thirty years of age in both the male and female.

In our study the percentage of malignant tumors in both the colored male and female is lower than that in the white male and female (Table 2). Pearl and Bacon (1927) said: "Malignant tumors of all sorts taken together occur from two to three times more frequently, relatively, among white persons than they do among colored persons." In considering all ages together (Table 2) in our study of autopsy cases on individuals twenty years of age and older, cancer occurred in 20.4 per cent of the autopsies on whites and 14.7 per cent of the autopsies on colored, a ratio of 1.38 to 1. This variation between our results and those of Pearl and Bacon (1927) may be influenced by the fact that cases of cancer of the breast and cervix have not been studied at autopsy in our institution as frequently as other malignancies. Permission for a post-mortem examination is not granted as readily when the primary site of the malignancy is known as when it is unknown. Tomlinson (1945) states that malignant tumors occur less frequently in Negroes than in whites.

The variation in the frequency of cancer in the different age groups in the white and colored races (Table 3) emphasizes the necessity for the study of the type of cancer that occurs in the two races. Since our present data are not suitable for a study of the specific types of neoplasms, we are now making such a study based on our autopsy cases during the past five years.

Summary

5,264 cases were autopsied from 1920 to 1949 on individuals 20 years of age and older. 929 cases examined during this period had a malignant tumor.

45.4 per cent of the total autopsies were on colored. 37.8 per cent of the cancer autopsies were on colored.

53.9 per cent of the total autopsies were on whites. 62.2 per cent of the cancer autopsies were on whites.

68.7 per cent of the total autopsies were on males. 69.9 per cent of the cancer autopsies were on males.

TABLE 3

Frequency of cancer in the male and female in the various age groups as studied at autopsy

Age Group	WHITE MALE			WHITE FEMALE			COLORED MALE			COLORED FEMALE		
	No. of Autopsies	No. of CA Cases	% CA	No. of Autopsies	No. of CA Cases	% CA	No. of Autopsies	No. of CA Cases	% CA	No. of Autopsies	No. of CA Cases	% CA
20–29	154	12	7.8	122	8	6.6	139	10	7.2	168	9	5.4
30–39	234	19	8.0	108	14	13.0	249	19	7.6	219	19	8.7
40–49	370	68	18.4	144	40	27.8	340	37	10.9	205	33	16.1
50–59	503	114	22.7	112	32	28.6	320	66	20.6	166	36	21.7
60–69	459	119	25.9	104	32	30.8	248	52	21.0	86	18	20.9
70–79	274	70	25.5	72	22	30.6	110	32	29.1	48	7	14.6
80+	73	13	17.8	28	5	17.9	25	7	28.0	12	1	8.3
Unknown	68	9	13.2	15	1	6.7	34	2	5.9	16	3	18.8
TOTAL	2,135	424	19.9	705	154	21.8	1,465	225	15.4	920	126	13.7

31.0 per cent of the total autopsies were on females. 30.1 per cent of the cancer autopsies were on females.

59.3 per cent of the total autopsies on males were on white males. 65.3 per cent of the cancer autopsies on males were on white males. 40.7 per cent of the total autopsies on males were on colored males. 34.7 per cent of the cancer autopsies were on colored males.

43.3 per cent of the total autopsies on females were on white females. 55.0 per cent of the cancer autopsies on females were on white females. 56.6 per cent of the total autopsies on females were on colored females. 45.0 per cent of the cancer autopsies on females were on colored females.

In this study cancer occurs more frequently in both the white male and white female than it does in the colored male and colored female.

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A HELMINTHOLOGIC SURVEY OF RAILWAY EMPLOYEES OF THE WARSAW RAILWAY CIRCUIT

ANNA STRYJECKA-USTUPSKA*

A large scale parasitologic survey was performed over the period 1945–1951 by the laboratories of the Railway Sanitary Services on a total of 5,019 specimens of human feces (46.88% from men and 53.12% from women) examined for ova of helminths. The age distribution was from 12 months to 75 years. Positive results were obtained in 22.25% of cases of which 11.80% concerned men and 10.35% women.

Infestation with the whipworm prevailed among men, while in women tapeworm was more common. The prevalence of *Trichuris trichiura* covered all age groups to 60; whereas *Ascaris lumbricoides* was prevalent in individuals up to 30 years of age in almost equal distribution among men and women.

Taenia solium appeared in men in the age group of 16 to 35, and in females from teen-age to 55. *Taenia saginata* affected men of 46 to 50 and women up to 40. *Oxyuris vermicularis* was recorded in men of 21 to 25, and in females from 6 to 25. *Diphyllobotrium latum* was detected in 7 cases over the period 1945–1949, but the age groups were not recorded.

As to the seasonal prevalence of helminth eggs, a distinct increase in the spring, mostly in March, and in the fall, mostly in October, was noted. A supplementary examination of 819 persons was made in 1951 including 59 persons from rural localities of the Warszawa region and from other areas. This type of rural survey is the first contribution to Polish helmintho-geography. It is of interest to note that of the 59 specimens from rural areas helminth ova were found in 3 cases only. Of the total of 819 fecal samples positive results were obtained in 13.78%. The folowing are the data for the workers of the Warsaw Railway district:

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*From the Chemical-Bacteriological Laboratory of the Sanitary Service of the Warsaw Railway Circuit. Chief of the laboratory, Dr. Taddeus Stzyjecki. Received for publication August 20, 1952.

Trichuris trichiura	in	564	cases (11.25%)
Ascaris lumbricoides	in	289	cases (5.76%)
Taenia solium	in	225	cases (4.41%)
Diphyllobotrium latum	in	7	cases (0.20%)
Oxyuris vermicularis	in	64	cases (1.28%)

As seen from the table, *Trichuris trichiura* is the most frequent parasite found among the railwaymen of the Warsaw district. This parasite appears in all age groups from 2 to 66 years and is more frequent in men than in women. *Ascaris lumbricodies* comes next attacking chiefly the age group from 1 to 10 years although it was detected 3 times in women of 66, 64 and 62 years of age. *Taenia solium* which occupies the third place (4.4%) was more frequent among women than men beginning with 15 years of age. *Taenia saginata* made a rare appearance (0.2%) which could be explained by the extensive consumption of pork as compared with the limited use of beef mainly by peasants and the working class. In other countries, *e.g.*, in France, *Taenia saginata* is by far the most widely spread parasite. The rarity of *Diphyllobotrium latum* in Poland is striking. Of a total of about 5,000 examinations made in the years following World War II, this parasite was found in 7 cases only (0.14%). Comparatively rare was the appearance of the pinworm (Oxyuris vermicularis) which is known to spread very easily.

These investigations occupy the second place as to the number of persons examined in Poland for infestation with helminths and are the first with respect to the uniformity of human material examined. One of the significant results is the lower prevalence of helminths among the people of Poland as compared with the findings of other Polish investigators before and after World War II. This points to the better understanding of hygiene and of sanitary measures by the individual than in the past. This is significant inasmuch as the Polish nation might have easily become a prey to the invasion of parasites under the German occupation, particularly the 1½ million inhabitants of Warsaw who were expelled from their homes, constantly driven from one place to another, badly clothed and poorly fed, with no fuel, soap, and often without water on their forced journeys.

SYMPOSIUM ON NUTRITIONAL FACTORS IN CANCER RESEARCH

Introduction

M. M. WINTROBE

Now that so much progress has been made in countering the attacks of various infectious diseases on the human population and in meeting the demands of deficiencies of different types, the importance of research designed to discover the cause and prevention of cancer becomes even more apparent than before. This is being recognized to an increasing degree, as indicated by the attention the subject is receiving, not only from those who have been avowed cancer students but also from investigators of greatly different interests. The result is that significant progress is being made in this field.

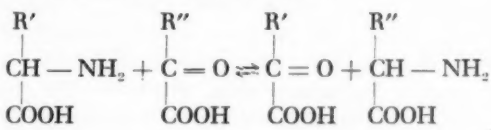
A fundamental feature of cancer is the growth of cells. This growth, however, is not the well ordered development of cells integrated with their environment and taking a useful place in the organism of which they form a part. Cancer represents abnormal growth which takes place entirely without regard to its environment—harsh, ruthless, overwhelming, undemocratic. It is logical, therefore, that we should seek to learn not only about the ways in which various nutritional substances are concerned in cell growth but also about the infinite details whereby growth occurs, since thereby we may hope to learn how normal cell growth becomes altered and results in the deleterious activity represented by cancer. It is also essential that we understand the factors which govern the development of cells.

We are fortunate in having been able to gather together for this symposium a number of investigators who have made important contributions to these fields of research. They have been asked to discuss their own observations and, in addition, to review related studies by others so that we may gain thereby a comprehensive picture of the whole field of nutrition in relation to cancer. It will become clear from these reports that much specific information has been gained concerning pure compounds as well as about less sharply defined substances which are carcinogenic, and also it will be shown that we now understand something of the role which nutritional and hormonal factors play in cancer growth. It is especially encouraging to see how much knowledge has been gained concerning the chemical processes which are concerned in cell metabolism and the mechanisms whereby cell growth can be influenced. Although much of our knowledge has come through animal and test-tube experimentation and even though we must be fully cognizant of the fact that the step from such observations to their application in man must be taken with due reservation and understanding, it is already clear that important advances in our approach to cancer in man have been accomplished through these means.

THE TRANSAMINASES AND FREE AMINO ACIDS OF HUMAN THYROID TUMORS*

JORGE AWAPARA

The search for some typical property of tumor tissue that may differentiate it from normal tissue has been an intensive one, even though unsuccessful. Nearly every possible cellular component has been measured; but to this day, there are no definite properties that can be ascribed to tumor tissue exclusively. The extensive work of Greenstein and associates on the enzymes of tumors has revealed that most tumor tissues resemble each other in their enzymatic pattern more than one tissue resembles another tissue. The study of enzymes in tumors is far from complete; and to add more negative data to the bulk of negative literature, I would like to present the results of this study on the transaminases of human thyroid tumors. The transamination reaction was discovered by Braunstein and Kritzman (1937), who observed that glutamic acid was utilized by tissue homogenates (liver and muscle) in the absence of oxygen and did not give rise to ammonia. On further study, they discovered that the amount of total amino nitrogen in the incubation mixture did not change. They inferred that the amino group from glutamic acid must be transferred to form another amino acid. The reaction as postulated by Braunstein and Kritzman could be expressed in general terms as follows:



Without going into the mechanism of this reaction, it may be stated that tissues contain an enzyme or group of enzymes that catalyze the transfer of amino groups from amino acids to some keto acids. A survey of compounds thus affected by this new reaction revealed, according to Braunstein, that all amino acids can enter into this reaction, but only few keto acids, namely,

*From The University of Texas, M. D. Anderson Hospital for Cancer Research, Houston. This work was supported in part by funds from the American Cancer Society.

∝-ketoglutaric acid, oxaloacetic acid, and perhaps pyruvic acid. Other investigators (Cohen, 1939, 1940; Cohen and Hekhuis, 1941) attempted to confirm Braunstein's results but were unable to do so. The transamination reaction was considered to be a reaction limited to the dicarboxylic amino acids and alanine. At this point, it may be pertinent to state that the analytical methods used to measure transaminases were not very specific or accurate. In recent years, the problem has been revised and all the evidence points to the fact that most tissues possess a large variety of transaminases (Cammarata and Cohen, 1950; Awapara, 1952). The transaminase activity of embryonic tissue, tumor tissue, and normal tissue has been measured in several laboratories (Braunstein and Azarkh, 1939; Cohen and Hekhuis, 1941). The majority of reports indicate that tumor tissue possesses lower transaminating activity than normal tissue, and in some instances the claim has been made that tumor is totally inactive. Cohen studied the effect of feeding dimethylaminoazobenzene on the transaminase activity of liver, and found that there is a progressive drop in activity. Embryonic tissue has been found to be less active than normal tissues. In general, transaminase activity appears to be decreased in rapidly growing tissues. The implications of this finding are very important inasmuch as transamination appears to be an important reaction in the nitrogen economy of most organs.

We began our work by surveying transaminases in rat organs and extended the investigation to human tumors obtained from surgery. Thyroid tumors were chosen as a matter of convenience and because of the general interest that exists here on this type of neoplasm. The technical difficulties associated with this type of experimentation minimizes the significance of the results, even though great efforts were made to eliminate as many variables as possible. The specimens investigated were obtained soon after surgical removal and sampling was performed within a short time. Several portions of what appeared to be "normal" thyroid tissue were removed and other portions of pure tumor also cut out of the surgical specimens. Each sample was then sectioned into two portions, one of which was used for histological examination, and the other portion was immediately frozen with dry ice. The latter sample was analyzed for free amino acids and transaminases within one hour

after the removal. Thus, many possible errors were eliminated. The results which are shown in the table indicate that there is little correlation between transaminating activity and malignancy. If the differences shown in the table are significant, then, tumor tissue has a higher transaminating activity than the normal tissue obtained from the same specimen. Were it not for the histological examination of the specimens, these figures would have been totally misleading. Dr. Jacob Old, from the Department of Pathology, kindly studied all the specimens and attempted an estimation of the various types of cells present. A better correlation was found when enzymatic activity was compared to the amount of active cells in the tissue. The colloid obviously is devoid of enzymatic activity (at least in regards to transamination), so that specimens of high colloid content had low transaminase activity; and specimens of low colloid content, but rich in active cells, were found most active. A similar correlation exists between colloid content and concentration of free amino acids. This is clearly seen in the chromatograms that represent free amino acids in the various samples. In every case the number and concentration of free amino acid is higher in samples where there is a larger number of cells.

TABLE 1

Transaminases in Thyroid and Thyroid Tumors

The Values Are Measured in Micromoles

Case No. and type	Glutamate Formed Per Gram of Tissue Per Hour				
	Aspartic	Alanine	Leucine	Isoleucine	Valine
Normal					
7252	33	20	4	13	...
7255	132	30	...	3	4
7256	85	15	...	...	...
7258	43	3	...	...	...
6921	58	8	1	6	...
Tumor					
6286	146	52	...	118	56
7252	99	...	...	...	...
7258	135	...	...	22	...
7454	111	2	...	16	9
S5320M	75	1	...	...	...
Hyperthyroid	202	40	25	37	27

Conclusions

The results of this study are negative in that they fail to correlate transaminating activity with "rapid growth." On the other hand, these results point to the fact that no biochemical study of "tumors" is reliable unless a parallel histological study is carried out in the same specimen. These results cannot be reconciled with the reports of other investigators that claim lower transaminase activity in rapidly growing tissue than in normal tissue. However, it would be futile to compare human thyroid tumors with induced hepatomas in the rat. If a certain amount of speculation is permitted, I would like to state that enzyme concentration in many tissues depends on a number of factors. For example, transaminases in the ventral lobe of the rat prostate depend on the state of that organ. Results, as yet unpublished, obtained in this laboratory, demonstrate that alanine transaminase in the ventral prostate drops to very low values after castration, whereas, aspartic transaminase remains almost unchanged. The loss of alanine transaminase runs parallel to the loss of tissue mass. This would indicate that some protein component with which alanine transaminase is associated is lost more readily than the other components of the prostate. A similar effect is induced by estrogen treatment. In this case, high transaminase activity is associated with rapid growth in contradistinction to results obtained elsewhere. Undoubtedly, the study of enzymes in tumors necessitates careful evaluation of results since there are so many variables that can lead to erroneous interpretations.

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VIRAL CONCEPTS OF HODGKIN'S DISEASE

WARREN L. BOSTICK*

The identification of a virus and its linkage to a known infectious disease is usually a long and difficult task. The relationship between cancer and viruses is especially challenging (Russell and Wynne, 1951). Although the small size of a virus is a major factor causing this difficulty, it is by no means the only one, for the simple reason that a virus is recognized, not by its size alone, but by a whole series of characteristics, no one of which is specific. The fact is that each characteristic is nonspecific, and it is only by the demonstration of several so-called "virus properties" that the existence of a possible virus can be surmised. In recounting the major properties that must be shown in order to establish the existence of a virus, certainly its ability to reproduce is most important.

Reproduction is usually most easily established by noting the persistence of, or increase in, a virus effect after great dilution. This can be demonstrated by various methods, such as serial passage of the virus on susceptible substrates, during which the effects of the virus are not diminished in spite of the great dilution of the original inoculum. This continuing effect is considered to have only one explanation—reproduction of the virus.

Having demonstrated that the supposed virus seems to multiply, it must next be shown to fall within certain size limits, generally being referred to as "filterable." This property places them in a size group below that of the bacteria, since the vast majority of viruses are smaller than bacteria and most of them are filterable. Filterability, however, is not a simple property to establish, since the virus may be too large to pass through the selected filter, or the virus when passing through the filter may be absorbed, inactivated, or destroyed by certain properties of the filter. The inability to filter a supposed virus factor, therefore, does not disprove its true virus status.

Of paramount importance in establishing the presence of a virus is its action. A virus must be able to do something that can

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be detected and preferably measured. In general, this action is directed against the living cells with or in which viruses, by definition, must grow. Generally, the effect of a virus on a cell results in an abnormal cell reaction and therefore a so-called "sick cell." The virus thus causes disease, and the presence of the virus is detected by the variably characteristic disease that it produces. If a virus does not at some phase of its existence cause a "disease" in the host cell, it in general cannot be detected, measured, or classified as a virus. Such non-disease-producing viruses must exist; however, they essentially completely escape detection by the present methods of virus study.

The cell abnormality produced by a supposed virus is of great importance. It may manifest itself by changes apparent only in the individual cell, in tissues, in the entire organism, or in any combination of them. Most frequently identified are individual cellular changes, such as inclusion bodies, cellular proliferation, and cellular lysis. The cellular lysis or proliferation is quite non-specific in appearance, but the inclusion bodies may be much more characteristic. The inclusion bodies may consist of abnormal masses of cytoplasm which have separated out due to the virus injury to the cell. Also, they may represent actual agglomerated colonies of the actual virus, such as elementary bodies. Sometimes the virus may be so large as to be seen individually or in groups of two or three. If the identity of cellular particles is in doubt, they may be referred to as "macromolecular particles." It is important to recognize that all of these types of virus masses are not morphologically pathognonmonic. When, however, they possess a constant staining property, exist in certain cells, and are noted in constant parts of a cell, such as the Negri body of rabies, these inclusion bodies may be highly specific and of great diagnostic aid when combined with proper clinical information.

In tissues and organisms, viruses characteristically result in an inflammatory reaction. This presents a broad spectrum of variation, but ordinarily it assumes the form of a chronic round-cell type of leukocytic response. Thus, prominent in the inflammatory reaction are lymphocytes, macrophages, plasma cells, and later fibrosis. A few giant cells may be present plus a variable degree of necrosis. The overall host reaction to this in-

jury manifests itself characteristically in the form of fever, chills, malaise, anorexia, and various localizing signs and symptoms depending upon the site of the main tissue injury. The peripheral blood count is variable; but more frequently than not, the leukocytosis is not striking; and, instead, there is a leukopenia. There may be a relative or absolute increase in the mononuclear white blood cells. This reaction of the entire host is again not specific, but is nonetheless characteristic enough so that when it occurs following exposure to a possible virus factor, it is evidence in favor of the factor being a true virus.

Perhaps the most important thing to establish about a possible virus disease is that it is infectious and can be transmitted and produce an identifiable disease. Without this property, all other viral characteristics seem to lose their significance. If the transmission is clinically obvious, there is no problem in this regard; however, various varieties of occult transmission exist and can make the property of the infectiousness most difficult to demonstrate. Several possible variants exist.

The supposed virus may be readily transmissible but may have a vastly greater clinically quiet "carrier rate" than obviously sick "morbidity rate." If such is the case, the transmissibility of the disease can easily be overlooked unless large numbers of susceptible people are exposed to the virus. This situation would become even more difficult to unravel if exposure to the virus resulted in a relatively permanent immunity. Much of the adult population would thus already be immune to the virus, and tests for its transmissibility would be most difficult. Under such circumstances, if the morbidity rate is low, it would be statistically practically impossible to detect an epidemic or to demonstrate case to case transmissibility. In many ways, poliomyelitis virus demonstrates these characteristics.

The transmission may be even more complicated. If the possible virus disease under consideration demonstrates the "vertical" transmissibility emphasized by Gross (1951), instead of the classical "horizontal" transmissibility, its infectiousness cannot be demonstrated without examining different generations of individuals for evidence of the virus. In the "vertical" type, a virus can only be passed directly from the parent (mother, usually) to the egg and embryo (Figure I). The offspring is

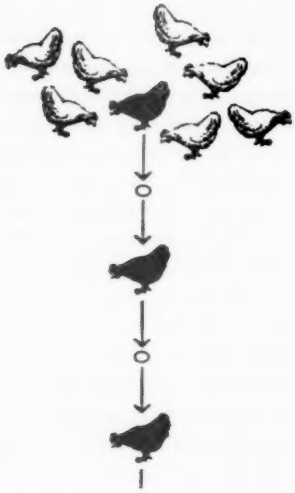

FIG. I. Vertical transmission of the avian lymphomatosis virus from generation to generation via the egg (after L. Gross).

born with the virus infection which may, after a variable incubation period, develop or be activated into a recognizable disease later in the life of the offspring. If the morbidity rate of this type of virus infection is low, few if any of these individuals will become clinically ill, and the virus infection may even not appear as a disease during that entire generation. The demonstration of the infectiousness of such a virus would be excessively difficult. In the fowl, infectious lymphomatosis seems to be such a disease (Cottral, 1950). In man the obstacles to demonstrating such a type of virus infection are essentially insurmountable.

If it is impossible to successfully transmit a suspected virus disease to the animal from which it was obtained, a major doubt will exist that one is indeed dealing with a virus. The above paragraphs dealing with vertical transmission and high carrier rates might explain the lack of success, but do not remove the doubt. A poor alternative exists. The supposed virus could be isolated consistently from the diseased primary host and then

inoculated into another, secondary species in which an identifiable disease might be produced. If the resultant secondary disease resembles that found in the primary host, the possible connection between the transmitted agent and the primary host's disease will be very strong. If, however, the resultant secondary disease bears no apparent resemblance to the original disease, the link of evidence is weak. All that can be concluded from such a result is that an infectious agent had been isolated. Repeoted isolation of the supposed virus from the primary host only when it showed the classical disease entity, and its repeated passage to an animal of different species with resultant development of a disease of different signs and symptoms would certainly increase the likelihood that the two disease processes were related, but would not prove it.

It is apparent, therefore, that the production and transmission of a characteristic disease due to a supposed virus are clearly of more fundamental importance in proving the existence of a virus than is demonstrating even several of the many physical properties of a virus organism. Until all of the requirements of Koch's postulates have been met regarding a possible infectious etiological virus agent, some doubt will remain regarding its specific characteristics even if, in every physical property, it possesses the characteristics of a virus.

In Hodgkin's disease the accumulation of data in favor of a viral etiological agent has been considerable. In man the clinical signs and symptoms have always been most suggestive of an inflammatory type of process. Pathologically this impression is supported by the characteristic pleomorphic cellular reaction. The typical Sternberg-Reed giant cells are of reticulo-endothelial origin and lie in lymphoid tissues distorted classically by fibrosis, plasma cells, granulocytes, and eosinophils. Focal areas of necrosis are not rare.

The search for inclusion bodies, elementary particles, and macromolecular elements of viral type has been extensive. Many workers have reported their presence. Gordon (1932) compared them with those found in vaccinia. The large nucleolus-like structure in the Sternberg-Reed cell has often been suspected as being an inclusion body equivalent. In cell-free and sterile Hodgkins' disease tissues, Grand 1949, Reiman *et al.* (1950),

Rottino (1949), and Hoster *ét al.* (1950) studied macromolecular particles. Their presence and physically acceptable characteristics are generally acknowledged. However, opinion is clearly divided as to their significance and their Hodgkin's disease specificity. Some believe them to be non-specific.

Animals of all types have been used to try to identify a specific disease or evidence of a virus type infection. In numerous species of adult animal experiments it has never been possible to demonstrate any evidence of Hodgkin's disease causing a specific reaction. Tissue cultures do seem to possess peculiar changes when exposed to Hodgkin's disease extracts (Rottino, 1949; Reiman *et al.*, 1950; and Grand, 1949). Again, however, the interpretation of their significance varies, with some authors concluding that they are quite characteristic and bona fide evidence in favor of a virus type of reaction, and others disagreeing.

More encouraging results have been obtained in Hodgkin's disease studies employing either embryos or very young animals for inoculations. It has long been known that the immune mechanisms of embryos and newborn animals are imperfect and that they may demonstrate susceptibilities to infectious agents which may be absent in the more mature animal. They may even become infected with organisms which are not normally pathogenic for their species. In Hodgkin's disease, the survey of these possibilities has only relatively recently begun (Bostick, 1952).

Embryonated chicken eggs have been inoculated with sterile and often filtered Hodgkin's disease extracts. Karnofsky *et al.* (1947) recorded that such injected embryos were often edematous and abnormal in appearance. Inoculation of Hodgkin's disease extract filtered and serially passed in chicken eggs resulted in an increased embryo mortality (Bostick, 1948) when compared with equivalently inoculated control embryos. This mortality was slight and would best be explained as a result of proliferation of a filterable agent. The embryos dying after the Hodgkin's disease inoculations showed no characteristic anatomical or histological changes.

In an effort to detect the presence of a possible viral agent in these Hodgkin's disease inoculated eggs, tests were made for the presence of virus interfering properties of the Hodgkin's

disease material. Primary amniotic inoculations of fertile chicken eggs with the serially passed Hodgkin's disease material were able to interfere frequently with the growth of influenza virus inoculated after three more days of incubation (Bostick, 1950). The conditions necessary to demonstrate interference are apparently very strict. Horsfall (personal communication) was unable to demonstrate any interference activity in chicken eggs under conditions only approximately similar to Bostick's.

Since the inoculated eggs show no structural evidence of disease, and thus observations on them cannot be positively related directly to the Hodgkin's disease material with which they are inoculated, attention has been directed to young animals. Newborn mice fail to show any abnormalities upon Hodgkin's disease inoculation. However, interesting responses are appearing in series of inoculated young guinea pigs.

Hodgkin's disease lymph node material is inoculated into the amniotic sacs of seven day embryonated chicken eggs. They are harvested routinely five days later. The harvested amniotic fluid is injected into a new group of eggs. Identically passed extracts of non-Hodgkin's disease lymph nodes (with metastatic carcinomas, etc.) are carried as controls. This serially passed and sterile harvested Hodgkin's disease amniotic fluid is inoculated sub-cutaneously, intramuscularly, intraperitoneally, and intranasally into each of a group of 250 gram (approximately) guinea pigs. The similarly passed control amniotic fluid is inoculated into another identical group of control guinea pigs. Their temperatures and blood counts are then followed for the subsequent five weeks. The rectal temperature charts of these animals are not remarkable. The total leukocyte count tended to rise in the first two weeks. The percentage of granulocytes in the peripheral blood of the Hodgkin's disease inoculated animals was greater than in the control inoculated groups on repeated occasions (Table 1, Figure II).

These experiments may well be demonstrating for the first time in mammals a detectable abnormal reaction to Hodgkin's disease material which has been processed in such a manner to support strongly the thesis that only a virus type agent could account for these observations. These observations must be amplified, the effect of other factors such as cortisone studied, and

TABLE 1

Mean Percentage, Polymorphonuclear Leukocytes in Guinea Pig Blood

	Control Animals	"Hodgkin's" Animals	Probability
Normal:			
2–13 days	21 ± 6.3%	21 ± 7.3%	50.00
"Hodgkin's" injected:			
1–3 days	18 ± 6.4	25 ± 6.9	0.01
4–8 days	19 ± 5.0	26 ± 4.5	0.01
2nd week	20 ± 6.0	24 ± 6.0	0.01
3rd week	21 ± 5.3	30 ± 9.0	0.01
4th week	22 ± 4.9	27 ± 9.6	0.05
5th week	23 ± 8.1	27 ± 9.7	0.15
Mean	21 ± 2.7	27 ± 2.5	0.01

detailed pathologic examination made to elucidate etiological relationships in Hodgkin's disease.

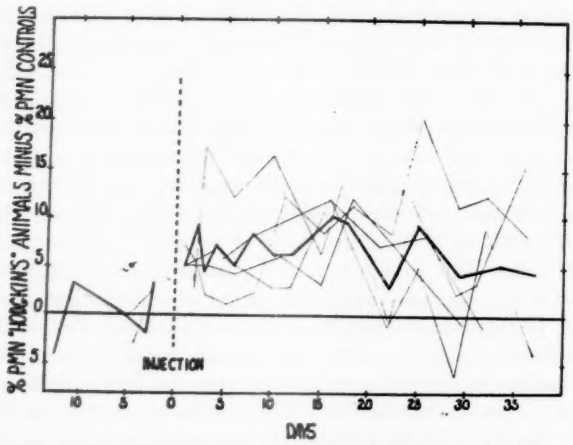

FIG. II. Graphic representation of data in Table 1, showing the granulocytosis that developed in the young guinea pigs following Hodgkin's disease extract injections. The heavy line is the mean for all of the determinations.

It is apparent that the studies on Hodgkin's disease have demonstrated many properties which are entirely consistent with this neoplasm being caused by a virus. However, the absolute evidence of its virus etiology is not yet at hand. The last necessary step is to reproduce successfully the disease in a typical

and identifiable form, preferably in man. If because of technical, sociological, or immunity reasons this is impossible, an especially important substitute can be made. Every effort must be made to establish this disease in another animal. The above-described studies on embryonic, newborn, and young animals are most important steps.

In spite of the present inability to fulfill Koch's postulates in establishing the virus etiological agent in Hodgkin's disease, it is true that an increasing mass of experimental data is pointing in favor of a virus type factor as being intimately associated with the cause of Hodgkin's granuloma.

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SERUM ACID PHOSPHATASE INHIBITOR

I. Effects of Dilution

Franklin T. Brayer and Jack B. Trunnell*

Of all the types of malignancy amenable to experimental study, metastic cancer of the male prostate gland is probably one of the most satisfactory because the serum acid phosphatase tends to fluctuate as a direct function of the activity of the disease. The investigator is thereby provided with a reasonably good index of effectiveness of therapy.

Unfortunately, only about 85 per cent of cases of metastatic cancer of the prostate exhibit serum acid phosphatase levels that are in keeping with the stage of the disease as shown by Sullivan, Gutman, and Gutman (1942). In the other 15 per cent, the serum levels of the acid phosphatase are low or within normal limits and tend to stay there in spite of progressive disease or therapeutic manipulations. The reasons for this discrepancy are not apparent.

Assuming that the phenomenon might be due in part to the presence of an inhibitor of serum acid phosphatase, the problem was attacked from the viewpoint of attempting to demonstrate such an inhibitor.

Goldstein (1943) and Straus and Goldstein (1942–43) have shown in a detailed experiment that the effect of dilution on an enzyme inhibitor sytem can be expressed as an inhibition of the inhibitor. Hussey and Northrop (1922–23), working with an enzyme inhibitor system, have observed that dilution resulted in dissociation of the enzyme inhibitor complex.

Procedure

All the patients studied, except the controls who had no disease, were histologically and radiologically proved cases of metastatic cancer of the prostate. They had all been either castrated and/or were receiving estrogenic therapy[1] while being

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[1]Ethinyl Estradiol (kindly supplied by the Schering Corporation, Bloomfield, New Jersey), 0.15 mg. daily, usual dose.

studied. Patients R. Z., W. H., and D. B. (Table 2) were exceptions to this statement in that they had had no treatment at the time they exhibited inhibition in their serum.

All determinations of the serum acid phosphatase activity were made in duplicate by the Woodard (1942) modification of the Bodansky (1933) method on fresh, fasting serum. The volume of serum plus substrate was kept at a total of 8 milliliters. The following amounts of serum were used:

1. 0.8 ml. serum plus 7.2 ml. substrate.[2, 3]
2. 0.4 ml. serum plus 7.6 ml. substrate.
3. 0.1 ml. serum plus 7.9 ml. substrate.

It can be seen then that the serum dilution factors involved in the observed alterations of enzyme activity in this study were 10, 20, and 80. Temperature of incubation was 37° C.; pH was kept between 4.5 and 5.5. There is a broad maximum of activity of the serum acid phosphatase in this range.

The enzyme activity in all cases is expressed in Bodansky units.

From trial experiments using 0.1 ml. differences, from 0.8 to 0.1 ml., it was found that three dilutions used yielded as much information as did eight graduated dilutions within the same limits.

Time of incubation was kept at appropriate multiples of two and eight of the original time of incubation for the 0.8 ml. fraction (*i.e.*, if two minutes was selected as the time of incubation for the 1:10 dilution, four minutes was used for the 1:20 dilution and 16 minutes for the 1:80 dilution). This selection of the time of incubation was in accordance with the procedure outlined by Woodard (1942).

Varying the time of incubation in those instances where inhibition was demonstrated did have an effect on the final result. Never, however, did the effect vitiate the demonstration of the inhibitor effect. This result was presumably due to the effect of the products of reaction on the rate of enzyme activity (Langmuir, 1916).

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[2]The concentration of substrate was such that it did not influence the rate of action of the enzyme.

[3]Substrate volume refers to unbuffered substrate plus volume of N/2 HCl (0.25–0.29 ml.) necessary to obtain proper pH.

It was determined that the variation in reproducibility of the method employed was as follows:

1. 0.2 Bodansky units for the 1:10 dilution.
2. 0.4 Bodansky units for the 1:20 dilution.
3. 1.3 Bodansky units for the 1:80 dilution.

Only significant values are included in Table 2.

Results

Forty-one determinations on the sera of seventeen different patients were done (Tables 1 and 2). An increase in activity of

TABLE 1

Results showing no inhibition of inhibitor by dilution in the serum acid phosphatase in cases of metastatic cancer of the prostate

Date	Patient	M.D.A. #	Results in B. U.* for amount of serum tested 0.8 ml.	0.4 ml.	0.1 ml.
4/6/51	H.F.	6580	9.2	8.5	9.9
4/9	"	"	7.4	8.1	8.1
4/10	F.B.	Control	0.1	0.4	0.3
4/11	R.K.	6071	0.9	1.7	2.1
4/12	H.F.	6580	3.4	2.6	4.1
"	J.B.T.	Control	0.1	0.2	0.2
4/13	H.F.	6580	6.4	4.3	2.2
4/16	"	"	3.1	0.9	0.6
"	R.Z.	6592	38.8	14.4	9.4
4/17	"	"	68.4	15.4	20.4
4/18	A.C.	5678	0.4	0.4	0.8
"	H.F.	6580	7.6	6.7	4.4
"	R.K.	6071	4.8	3.9	2.4
"	R.Z.	6592	84.8	74.4	55.5
4/19	"	"	32.0	30.7	28.7
4/20	R.K.	6071	3.8	1.7	1.8
4/23	"	"	6.4	5.5	7.0
5/4/51	H.F.	6580	7.2	5.1	6.1
6/5	R.F.	Control	0.05	0.02	0.01
9/11	T.H.	2952	1.1	1.2	1.1
10/4	J.B.	5211	4.0	2.9	1.4
10/9	W.K.	7332	0.5	0.2	0.1
10/16	R.Z.	6592	0.6	0.2	0.4
1/17/52	D.B.	7123	70.8	69.7	65.0
"	J.D.	7071	0.4	0.4	0.4
1/29	W.K.	7332	0.2	0.3	0.3
"	L.M.	7233	0.2	0.2	0.1
1/31	J.B.	Control	0.1	0.2	0.08
2/5	J.M.	Control	0.2	0.04	0.03
"	J.T.	Control	0.3	0.4	0.1

*Abbreviation for Bodansky Units.

acid phosphatase with increasing dilution was demonstrated in eleven different instances in the sera of six different patients (Table 2). In no instance was a similar effect demonstrated in the sera of the six controls (Table 1).

TABLE 2

Results showing inhibition of inhibitor by dilution in the serum acid phosphatase in cases of metastatic cancer of the prostate

Date	Patient	M.D.A. #	Results in B. U. for amount of serum tested with range of reproducibility 0.8 ml.	0.4 ml.	0.1 ml.
4/9/51	R.K.	6071	1.8±0.2	1.9±0.4	3.4±1.3
4/10	H.F.	6580	3.3 "	4.2 "	5.3 "
4/11	"	"	4.8 "	6.1 "	5.6 "
4/20	R.Z.	6592	3.6 "	5.4 "	3.9 "
4/23	"	"	1.6 "	11.4 "	12.6 "
4/24	"	"	13.4 "	15.9 "	24.9 "
4/26	"	"	19.8 "	22.5 "	21.5 "
7/10	R.K.	6071	16.1 "	12.5 "	19.9 "
9/18	W.H.	7030	4.8 "	12.4 "	9.5 "
9/20	D.B.	7123	352.8 "	463.2 "	352.8 "
"	J.B.	5211	2.8 "	6.0 "	13.2 "

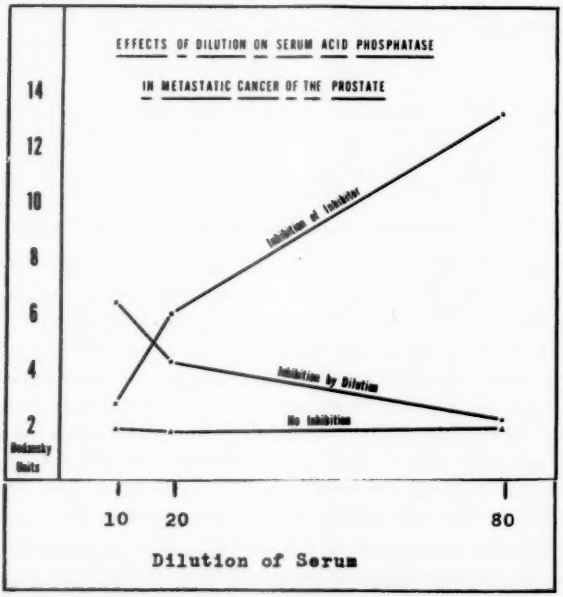
If no inhibitor for serum acid phosphatase were present (Graph I), then when enzyme activity was plotted against enzyme dilution factor, a horizontal line was obtained. On the other hand, if an inhibitor was present and was itself inhibited by dilution, a line showing an increasing slope to a maximum was obtained. In some instances, the maximum was evidently reached before the highest dilution, whereas in other instances it was probably not reached even with the highest dilution tried. Finally, if dilution brought about inhibition of the enzyme itself, then a line with a decreasing slope was obtained.

It might be mentioned that the alkaline phosphatase measurements which were done concomitantly with the acid phosphatase showed an occasional dilution effect, but it was not consistent and seemed to bear no relationship, whatsoever, to the results manifested in the acid phosphatase determinations. Hence, these data have been omitted from this presentation.

Discussion

Most of the available data concerning inhibitors of acid phosphatase have been obtained from *in vitro* studies. Various

materials such as oxalates, phosphates (Belfanti, *et al.*, 1935); arsenates (Sizer, 1942); fluoride (Kutscher and Wust, 1941); cysteine (Mattocke and Holtan, 1949); and a number of others were added to phosphatase-containing material, and were observed to depress the activating thereof. Such experiments always assume that the materials tested are normally present in the body and that the amounts used are in the same concentration as exists in the body. More often than not, both of these assumptions are difficult to prove.



GRAPH I. Three different slopes obtained from the data, showing inhibition of inhibitor by dilution, positive slope; inhibition of the enzyme by dilution, negative slope; and no inhibiton, zero slope.

In this experiment, the assumption was made that a naturally occurring inhibitor of serum acid phosphatase exists in the body in certain cases of metastatic cancer of the prostate. The results of the experiment bear out this assumption.

However, the results do not support the working hypothesis that the presence of this inhibitor is limited to the 15 per cent of cases of metastatic carcinoma of the prostate that do not show an increased serum level of acid phosphatase.

Neither from the data presented can any definite statements as to the effect of the stage of the disease or the stage of treatment, castration, and/or estrogens, on the amount of inhibitor present be made. There is some suggestion from a study of the clinical course of the patients followed that, as the disease regresses on therapy, there is a decrease in the amount of demonstrable inhibitor.

The data actually raise more questions than they answer. What is this inhibiting material? Is this inhibitor present in all patients at some stage of their disease, or is it present only in certain individuals, and if so, why? These and many more questions that could be raised will have to await further investigation. Some of these investigations are already in progress in this laboratory.

Conclusions

Data have been presented which suggest the presence of an acid phosphatase inhibitor in serum by means of a dilution technique.

It is concluded that the data obtained do not permit explanation of normal phosphatase levels in 15 per cent of cases of prostatic cancer in terms of the inhibitor herein described.

Acknowledgment

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ROLE OF FOLIC ACID AND CITROVORUM FACTOR IN METABOLIC PROCESSES AND THEIR RELATION TO CANCER

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Recent investigations in the folic acid field are rapidly extending our knowledge of the role of folic acid in metabolic processes and have given a clearer indication of the chemical nature of the actual folic acid coenzyme involved. It is pertinent to review present knowledge in the folic acid field in any discussion on normal or abnormal metabolism.

The description of Sauberlich and Baumann (1948) of an unidentified growth factor required by *Leuconostoc citrovorum*, termed the citrovorum factor (CF), and the subsequent findings by a number of investigators (Bond, Bardos, Sibley, and Shive, 1949; Broquist, Stokstad, and Jukes, 1950) that the CF was closely related chemically and biologically to pteroylglutamic acid (PGA) have called attention to the existence in natural materials of other forms of folic acid of biological interest. Chemical investigations have shown (Shive, *et al.*, 1950; Brockman, *et al.*, 1950) that a synthetic compound, variously termed leucovorin or folinic acid-SF, prepared by the reduction and formylation of PGA possesses the biological properties ascribed to the CF. The structure of this compound, resulting from chemical studies by several groups (May, *et al.*, 1951; Pohland, *et al.*, 1951; and Cosulich, *et al.*, in press), is 5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid as is shown in figure I.

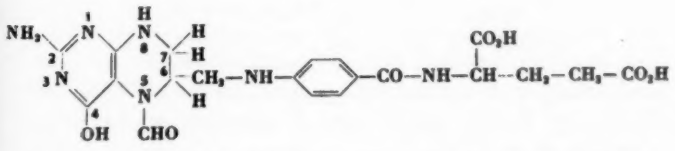

FIG. I. 5-Formyl-5, 6, 7, 8 tetrahydropteroylglutamic acid (Leucovorin, Folinic acid-SF).

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A summary of the microbiological activity of leucovorin, 5-formyl-5, 6, 7, 8-tetrohydro-PGA, and related compounds on several lactic acid bacteria is shown in Table 1. It is seen that

TABLE 1

Comparative Growth Response of "Leuconostoc citrovorum, Streptococcus fecalis and "Lactobacillus casei" to Leucovorin and Related Compounds

Organism	Amount of compound required per ml. culture medium for half maximum growth in 24 hours		
	Leucovorin	Acid-treated Leucovorin*	Pteroylglutamic Acid
	mγ	mγ	mγ
Le. citrovorum	0.15	7.5	30,000
S. fecalis	0.37	0.38	0.18
L. casei	0.17	...	0.09

*Prepared by allowing a leucovorin solution (4 mg/ml.) to stand at pH 2 for 24 hours at 25° C.

leucovorin is active as a growth factor both for *Le. citrovorum* and the folic acid requiring organisms *Streptococcus fecalis* and *Lactobacillus casei*, but that *Le. citrovorum* differs from the latter organisms in that it responds to PGA in only extremely large amounts. It is apparent, then, that when *S. fecalis* or *L. casei* are used to determine the folic acid content of natural materials these assays give a figure representing the sum of the PGA and CF present. Independent assay with *Le. citrovorum* will determine that proportion of folic acid existing as CF. There is now a good deal of evidence that the CF in many natural materials exists as a conjugate, which behaves as a polyglutamate analogous to that of PGA (Hill and Scott 1951).

It will be noted from Table I that exposure of CF to mild acid destroys essentially all of the activity (98%) for *Le. citrovorum*, but does not alter the activity for *S. fecalis* or *L. casei*. This acid degradation product of CF has been shown to be a new type of tetrahydropteroylglutamic acid derivative, in which an imidazoline or imidazolidine ring has been formed by a methylene bridge linking the N^5 and N^{10} position of the molecule (Cosulich, *et al.*, 1951).

Just as leucovorin can meet the requirement of certain microorganisms for PGA, it has also been found that leucovorin is active in promoting growth and hemopoieses in PGA-deficient chicks and turkeys (Broquist, *et al.*, in press; Hill and Briggs,

1951). These experiments suggested trial of leucovorin in anemia, and there is now considerable evidence that leucovorin, like PGA, is a potent hemopoietic agent in pernicious anemia, sprue, and the megaloblastic anemias of infancy and pregnancy.

It would appear from all available evidence published that before PGA can carry out its catalytic functions in the cell, it must first be converted to CF, and that CF either represents the catalytically active form of PGA or is more closely related to this form than PGA itself. This is supported by the finding of Nichol and Welch (1950) that there is an enzyme(s) in liver which can carry out this conversion. There is considerable evidence now at hand suggesting that PGA (and presumably CF) catalyzes the formation of several amino acids including serine, methiosine, and histidine and the purine and thymine moietics of the nucleic acids. Isotope studies from many laboratories have established that "formate" or its biological equivalent is incorporated into these metabolites. Thus, the knowledge that CF contains a formyl group is of considerable biochemical interest, because it provides a structural basis for the concept that CF is concerned with the transfer of the one carbon unit in metabolic reactions. If CF functions by shuttling a formyl group from one substrate to another, it might be expected that tetrahydro PGA might have activity comparable to CF. In support of this concept, it has been found (Broquist, *et al.*, 1951) that tetrahydro PGA has about 2.5% of the activity of leucovorin for *Le. citrovorum* and about one-third the activity of leucovorin in counteracting aminopterin toxicity in the mouse.

In view of the studies of 4-aminopteroylglutamic acid (aminopterin) and its congeners in cancer chemotherapy, the finding that the toxicity of aminopterin for growth of *Le. citrovorum* can be reversed competitively by CF (Broquist, Stokstad, and Jukes, 1950) is of considerable interest. From these data it may be postulated that aminopterin functions as a growth inhibitor by blocking those reactions in the cell which are catalyzed by CF. That aminopterin may function also in blocking the formation of CF has been shown by Nichol and Welch (1950), who have found that the antagonist blocks the *in vitro* conversion of PGA to CF by liver slices.

It was noted in Table 1 that leucovorin was about half as active as PGA for growth of *S. fecalis* and *L. casei;* however, if aminopterin is present in the medium, leucovorin is now markedly more effective than PGA in reversing the growth inhibition. This is illustrated in figure II. Such data imply that

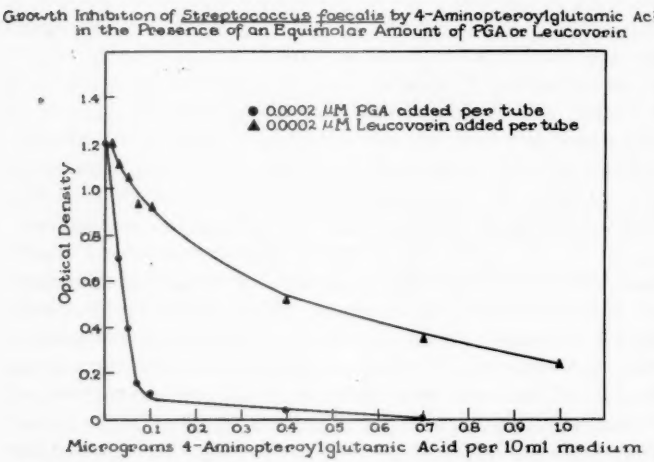

FIG. II. Growth Inhibition of *Streptococcus faecalis* by 4-Aminopteroylglutamic Acid in the Presence of an Equimolar Amount of PGA or Leucovorin.

CF may be more closely related to the biologically active form of the folic acid coenzyme than PGA itself. The finding that CF can reverse the toxicity of aminopterin for microorganisms is paralleled by numerous studies showing this effect in mice (Broquist, Stokstad, and Jukes, 1950), rats (Nichol and Welch, 1950), the chick embryo (Cravens and Snell, 1950), Drosophilia (Goldsmith and Harnly, 1951) and human beings (Schoenbach, Greenspan, and Colsky, 1950). Studies with mice have shown (Broquist, *et al.*, in press) that the amount of CF required to counteract aminopterin toxicity is approximately the same as the amount of antagonist employed; but if administration of the antagonist precedes the CF by even an hour, a considerably larger amount of CF is required to block the toxicity of the antagonist. The interesting phenomenon, observed by Greenspan

and associates (1951), that PGA may reverse aminopterin-toxicity in mice if the administration of PGA precedes the administration of the antagonist by a sufficient time interval is probably caused by enzymatic conversion of the administered PGA to CF, which is then able to compete and reverse the aminopterin.

Since one of the striking features of a nutritional deficiency of folic acid is the marked leucopenia which accompanies it, whereas the leukemias are diseases frequently characterized by excessive proliferation of leukocytes, the testing of analogs of folic acid, particularly derivatives of aminopterin, to combat these diseases has been widely investigated. An excellent review of the current clinical status of the antifolics in leukemia is to be found in the Proceedings of Second Conference on Folic Acid Antagonists in the Treatment of Leukemia (1952). Perusal of this report indicates that approximately 50% of all leukemic children given folic acid antagonist therapy experienced temporary clinical improvement, which usually lasted for a period of several months up to a year or two in isolated instances; the incidence of remissions was greater than that of spontaneous remissions. Quite recently, Swendseid *et al.* (1951) have shown that the folic acid content of leucocytes in leukemia is greater than in normal leucocytes. Moreover, the increase was correlated with the degree of cell immaturity, the highest leukocyte folic acid values being found in the acute forms of the disease. Thus, the beneficial effects of antifolic acid therapy may be caused by the greater need of the rapidly proliferating leukemic cell for metabolites arising by reactions catalyzed by folic acid (presumably the CF), and by the fact that the antagonist interferes with the functioning of CF. Use of antifolic acid therapy must proceed cautiously, because the amounts needed to produce a beneficial effect may approach levels that are toxic to the patient. These toxic effects are believed to be caused by the inducement of a severe folic acid deficiency. It has now been demonstrated clinically that the toxicity of aminopterin or amethopterin, as judged by the production of mouth lesions, can be alleviated without withdrawal of the drug if an adequate amount of leucovorin is administered (Schoenbach, Greenspan, and Colsky, 1950). The administration of leucovorin in these in-

stances may also counteract the beneficial effects of the folic acid antagonist. This was shown by the report of Burchenal, *et al.*, 1951; that they noted that the antileukemic effect of amethopterin in mice is reversed by the simultaneous administration of CF.

Another problem attendant to the use of the folic acid antagonists in leukemia patients is that if remissions are obtained, after a comparatively short time the disease becomes resistant to further therapy. A situation similar to that in patients can be produced experimentally in transplanted mouse leukemia, and it has been proved that the resistance to the antagonist is a characteristic of the neoplastic cell rather than of the cells of the host animal (Burchenal, *et al.*, 1951). In an attempt to gain a greater insight into the resistance a living cell can acquire to a folic antagonist, Burchenal, Waring, and Hutchison (1951) have produced a strain of *Streptococcus fecalis*, which is made resistant to amethopterin by successive subculture in the presence of increasing amounts of antagonist. This resistant culture can utilize either PGA or aminopterin for growth purposes. Preliminary experiments from this laboratory indicate that this resistant strain of *S. fecalis*, unlike the parent strain, has an enzyme(s) that can convert aminopterin to CF. Thus, when resting cells of the organisms are incubated under appropriate conditions with aminopterin, a substance is formed that is active for growth of *Le. citrovorum*. It thus appears that, in the process of becoming resistant to the folic acid antagonist, the organism developed the ability to transform the antagonist into the normal metabolite. Such studies throw some light on the mechanism of resistance to aminopterin in a microorganism. It is possible that a similar mechanism exists in leukemia cells which are resistant to folic acid antagonist therapy.

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METABOLIC AND CHEMOTHERAPEUTIC STUDIES WITH PURINE DERIVATIVES

GEORGE BOSWORTH BROWN*

Nucleic acids are not dietary essentials, and they are not known to play any specific role in mammalian nutrition. However, sporadic attempts have been made to administer nucleic acids, or products derived from them, for therapeutic purposes; but no conspicuous successes have attended those trials (Jackson, Parker, and Taylor, 1932; Reznikoff, 1930; Spies, Bean, and Vilter, 1940; Stone and Spies, 1948; Parsons, Gulland, and Barker, 1947; Daft, 1952; Albaum, Cayle, and Shapiro, 1951).

From the present metabolic data, it is known that orally administered intact pentose nucleic acid, such as might normally be encountered in the diet, furnishes a precursor for the tissue nucleic acid pyrimidines (Roll, Brown, DiCarlo, and Schultz, 1949). This is in distinct contrast to the fact that when the individual free pyrimidines are present in the diet they are not so incorporated (Plentl and Schoenheimer, 1944; Bendich, Getler, and Brown, 1949). Exactly the reverse situation pertains with the purines since those of orally administered intact nucleic acid are not utilized to an appreciable extent while certain free purines, notably adenine (Brown, Roll, Plentl, and Cavalieri, 1948), can be extensively utilized for the synthesis of tissue nucleic acids.

Although the purine adenine may be utilized for nucleic acid synthesis, large doses of it are partially oxidized to 2,8-dioxyadenine (Nicolaier, 1902; Bendich, Brown, Philips, and Thiersch, 1950), crystals of which are deposited in the kidney tubules and result in an appreciable toxicity. It may be significant that the adenine bound in ribosidic linkage in nucleic acids, when administered orally, is not available for tissue nucleic acid synthesis, and that massive oral doses of intact nucleic acid do not lead to the 2,8-dioxyadenine deposition and the concomitant kidney damage (Goldthwait and Brown, unpublished). Thus,

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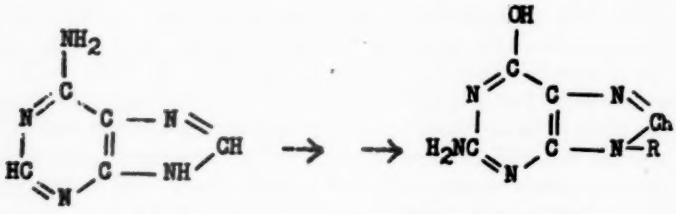
from the standpoint of this toxicity, the administration of relatively large amounts of nucleic acid is not contra-indicated. However, neither is it particularly suggested by the present knowledge of nucleic acid metabolism.

The circumstantial evidence, which suggests that the nucleic acids of the cell are critical factors in processes of growth and in the determination of the characteristics of growth, has led us into a study of the larger molecules which can be utilized as precursors of the nucleic acids. In parallel to this, and in cooperation with various other groups in the Institute, there has been a broad program of biological testing of the "antimetabolite" potentialities of a number of structural analogs of those substances found to serve as precursors. The present status of that aspect of our program is summarized here.

The purine adenine is not only incorporated as adenine but can also serve as a major source of nucleic acid guanine (Fig. I). From double labeling of the molecule (Marrian, Spicer,



Adenine Polynucleotide guanine

FIGURE I

Balis, and Brown, 1951), and other data (Balis, Levin, Brown, Elion, Vanderwerff, and Hitchings (1952), it appears that this transformation is accomplished with retention of the intact purine ring and that it must thus involve the introduction of a functional group in place of the hydrogen in the 2-position of the ring. From the standpoint of chemotherapy, any unusual substitution in that 2-position of adenine might well lead to a compound which could block normal metabolic reactions which must take place there. Because of its ubiquitous occurrence in many biologically important compounds, adenine is also a generally favorable target for any structural analogs of potential antimetabolite activity. In addition to being incorporated into

polynucleotides and to being converted into polynucleotide guanine, it is rapidly incorporated into the ATP fraction of liver (Marrian, D. H., unpublished), and to a small extent into muscle ATP (Brown, Roll, Plentl, and Cavalieri, 1948). It is also found in several of the coenzymes, but it has not yet been possible to study its incorporation therein. It is catabolized to urinary allantoin, both directly and via the nucleic acid purines. Excessive amounts of adenine can be oxidized without any cleavage of the ring to lead to 2,8-dioxyadenine (Bendich, Brown, Philips, and Thiersch, 1950).

In a study of several purines (Brown, 1948) which might conceivably be intermediates in the transformation of adenine into polynucleotide guanine it was soon found that 2,6-diaminopurine was another purine which was efficiently utilized and, in the case of the rat, it served as a precursor of polynucleotide guanine only (Bendich, Furst, and Brown, 1950). 2,6-diaminopurine has not been shown to occur in nature and it is simply a 2-amino substituted adenine. It was thus also a candidate antimetabolite. In fact before the metabolic experiment was completed, it was reported (Hitchings, Elion, Falco, Russell, Sherwood, and Vanderwerff, 1950; Elion and Hitchings, 1950) that 2,6-diaminopurine could competitively inhibit the utilization of adenine by *Lactobacillus casei*, and in our chemotherapy division it was demonstrated that it was toxic to mammals (Philips and Thiersch, 1949) and that it could influence (Burchenal, Bendich, Brown, Elion, Hitchings, Rhoads, and Stock, 1949) certain mouse leukemias, subsequently, some score of reports of varied inhibitory effects of diaminopurine have appeared, including specific effects on bone marrow and intestinal mucosa, tissues in culture, and viruses, as well as microorganisms. It has frequently been possible (except in the intact mammal) to demonstrate a specific effect of adenine in reversing these inhibitory activities.

We have also investigated the metabolic fate of all the purines which might be expected to be implicated in nucleic acid synthesis and are now studying those substances of the next higher levels of complexity, the purine ribosides (or nucleosides) and the phosphate esters of the purine ribosides (or mononucleotides).

The work of Dr. John Davoll in our laboratory resulted in a new general synthesis for purine nucleosides (Davoll and Lowy, 1951) by which it was feasible to synthesize C^{14}-labeled samples of several nucleosides in which we were interested. Since the total synthesis of nucleotides is not yet practical, such phosphorylated derivatives have only been prepared by biosynthesis. By the use of growing yeast (Kerr, Seraidarian, and Brown, 1951), the labeled adenylic and guanylic acids have been made available.

For the purposes of this discussion, the purine derivatives now known to function as the precursors of the polynucleotide purines in the rat may be summarized as in figure II. In that

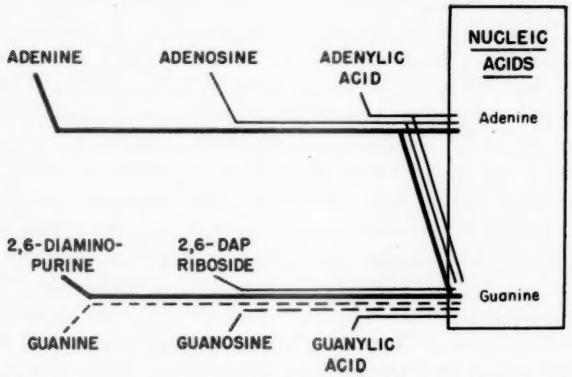


FIG. II. Pattern of incorporation of purines, their ribosides and ribotides into the nucleic acids of the rat.

diagram the heavy lines indicate extensive incorporation, that is, with low dilution factors encountered. The lighter solid lines indicate appreciable incorporation but with higher dilution factors and the broken lines indicate mere trace incorporation. There are individual lines for each over-all conversion, and no implication of a sequence of reactions is to be implied. It can be seen that all of the adenine derivatives lead to polynucleotide adenine and to polynucleotide guanine. However, 2,6-diaminopurine, its riboside, and the guanine derivatives are almost exclusively converted into polynucleotide guanine. The fact that

guanylic acid is much more readily incorporated (Roll and Weliky, 1951) into polynucleotides than is guanine or guanosine suggests that the conversion of an adonine derivative into a guanine derivative may well be taking place after attachment of at least the ribose.

It must be emphasized that this scheme represents the results in the Sherman rat, which is the only mammal for which we have this much data. In other species, mostly microorganisms, there are pronounced differences in the purine metabolism pattern, which range from this through complete interconversion of the two purines (Balis, Brown, Elion, Hitchings, and Vanderwerff, 1951), and to cases where guanine is converted into adenine but not vice versa (Flavin and Graff, 1951).

In parallel with the program of synthesis of C^{14}-labeled nucleosides for metabolic studies, over a score of nucleosides were made available among which were several variations (Davoll and Lowy, 1952) of potential interest as antimetabolites. There were a number of 2-substituted adenosines, as well as other variations in the purine ring, and several variations in the 9-glycosyl moiety. Derivatives of glucose and the "unnatural" pentoses, arabinose and xylose, were included, and ribopyranosyl as well as the natural 9-β-D-ribofuranosyl derivatives. Biological testing of this series has yielded several interesting results.

In considering the biological testing, it is perhaps more interesting to survey the response of a large number of structurally related compounds in *one* biological test. Dr. John Biesele has studied the effects of a large number of pyrimidines, purines, and nucleosides on bits of mammalian tissue in roller tube culture and has assessed the microscopically visible morphological damage produced in both normal and neoplastic tissues. Mouse embryo skin and Crocker sarcoma 180 have been the test tissues for a large series of purines and their derivatives. The results from some hundred substituted purines, submitted by a number of laboratories, have now been summarized (Biesele, Berger, and Clarke, 1952). Among all of them, certain adenine derivatives, with a simple substituent in the 2-position, have shown both the greatest toxicities and the greatest differential toxicities.

Among the adenines substituted only in the 2-position, 2,6-diaminopurine and 2-chloroadenine were far more effective since they were active at 0.1 and 0.05 mM, while 2-iodo, 2-dimethylamino-and 2-methyl-adenines produced similar damage at 2.0 mM. Some of these 2-substituted adenines were no more active than certain other purines, but the most effective compounds were in this group, and the percentage of interesting results was higher since five out of 15 were rated triple-plus in differential activity. In nearly all cases, there was a preferential damage to the neoplastic tissue, and in general the smaller and simpler 2-substituents were more effective. In the case of 2,6-diamino-9-methyl purine, a triple plus was obtained; but among 81 other variously substituted purines, only nine were rated as much as double-plus.

Among the 28 purine nucleosides tested, only six proved to have significant effects on the tissues in culture. These are shown in figure III. 2-chloroadenosine is the most toxic of the nucleosides; it evidenced a toxicity at one-tenth the concentration necessary with the other nucleosides. Four which showed

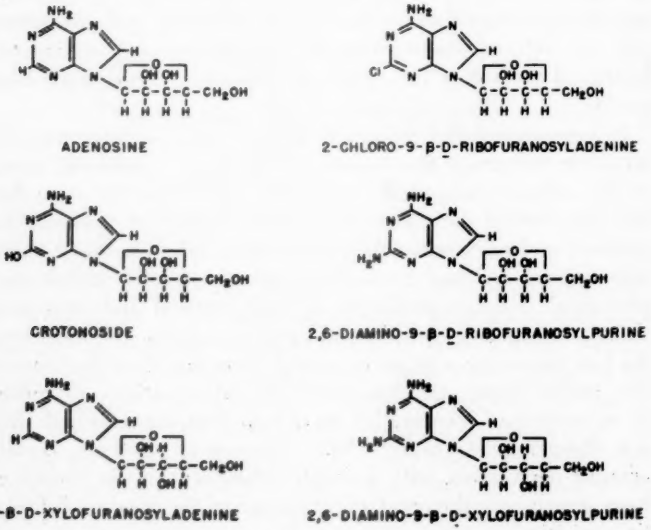


FIG. III. Nucleosides which show effects in tissue culture.

some effects were 2-hydroxyadenosine (crotonoside), 2,6-diaminopurine riboside, and two xylosides of adenine and diaminopurine (in which the configuration of carbon 3 of the pentose is altered). Of these, the diaminopurine riboside was least toxic, but it was the only one which showed a favorable differential between tumor and normal. Adenosine also produced a preferential damage to the Sarcoma 180 but required a longer time to do so. None of the ribopyranosyl, glucopyranosyl, or arabofuranosyl derivatives showed more than slight effects, and it may be pertinent that the only alterations of the "natural" pentofuranosyl-portion which were effective were those which retained the same configuration on the first and second carbons of the pentose moiety. It must be emphasized that, although the structural implications are interesting, the toxicities of all these nucleosides are far less than those produced by some of the purines, with the greatest differential damage rated as only single plus.

The blocking of the inhibitory effects of these compounds by various natural analogs was also studied. The inhibitory effects of either 2-chloroadenine or 2,6-diaminopurine can be blocked by adenine in a competitive manner, but are not blocked by adenosine or by other purines. However, the inhibition by 2-methyl adenine was not counteracted by adenine and this difference, plus the facts regarding the utilization of diaminopurine, raises the question of whether a similar mode of action is involved for all of these compounds. Among the polysubstituted adenines, 2,8-dichloroadenine is not toxic and also will not block the inhibition by 2-chloroadenine, and although the 2,8-dichloro-9-glycosyladenines were inactive, surprisingly enough the 2,8-dichloro-9-methyladenine shower some toxicity.

In the nucleoside series, the tissue damage produced by 2-chloroadenosine can be blocked by adenosine and not by adenine. However, in this instance, adenine does partially block the effect on the S 180 only, and so reduces the differential between it and the normal. The toxicity of diaminopurine riboside can be blocked by both adenosine and adenine. This might be rationalized by assuming that adenine and adenosine are both metabolically converted into the actual reversing agent. The additional fact that guanosine was found to potentiate the

toxicity is more difficult to explain, but may be related to diaminopurine riboside's role as a precursor of polynucleotide guanine.

The extension of the testing from tissue culture to the whole animal is complicated not only by the scarcity of the nucleosides but also by the results of an entirely different type of biological test in which the 2-substitution proved to play an important role. With Doctors Clarke and Philips, an investigation of the effects of various structural variations on the vaso-depressor activity of adenosine was carried out (Clarke, Davoll, Philips, and Brown, 1951). The results established the necessity for the presence of an amino group in the 6-position in the purine ring and for the "natural" β-D-ribofuranosyl configuration in the 9-substituent. In most cases where a 2-substituent was also present, the effects on blood pressure were notably enhanced.

Figure IV presents the maximum ranges of the relative vasodepressor activities and the relative duration of the depression

FIG. IV. Adenosine analogs with depressor activity.

Compound	Relative Depressor Potency			
	Activity	Duration*	No. Tests/ No. Cats	Qco_2†
Adenosine	1	1		6.00
2-Acetamido-Adenosine	0.4–0.8	5–29	5/3	0
2-Amino-Adenosine	0.5–1	1–3	10/4	2.64
2-Methyl-Adenosine	3–13	9–12	4/3	0
2-Hydroxy-Adenosine	5–19	4–6	5/3	0
2-Chloro-Adenosine	19–200	7–13	18/7	0

*To return to one-half initial pressure.
†MM.$^3CO_2$ consumed/hr./mgm. tissue wet weight (rat kidney homogenate).

for the series of 2-substituted adenosines. Diaminopurine riboside is slightly less effective but slightly longer acting than adenosine. Among the other 2-substituted adenosines which showed enhanced vasodepressor activity, 2-chloroadenosine evidenced the maximum activity of the series, with a vasodepressor potency approaching that of acetyl choline and with a greatly prolonged action. 2,8-dichloroadenosine, with the additional substituent on the 8-carbon, the 6-hydroxy nucleosides inosine and guanosine, and all variations of the carbohydrate moiety were without appreciable activity.

The susceptibility of this series of nucleosides to the action of adenosine deaminase preparations from blood and tissues was also investigated (Col. 5, Fig. IV). In addition to adenosine, 2,6-diaminopurine riboside was the only other nucleoside which proved to be deaminated by the preparations, and the fact that it can be deaminated correlates with its transitory effect upon the blood pressure. None of the 2-substituted adenosines which showed enhanced and prolonged effects on blood pressure inhibited the deamination of adenosine, and this fact suggests that their vasodepressor effects are characteristic of the compounds themselves and are not due to interference with normal metabolism of adenosine.

Before turning to another study in which this series of nucleosides was used, some studies (Balis, Levin, Brown, Elion, Vanderwerff, and Hitchings, 1952) of the metabolic fate of 2,6-diaminopurine in *L. casei* should be mentioned. Elion and Hitchings (1950) had found that diaminopurine could inhibit the growth of *L. casei* and that adenine could specifically block this inhibition. They also found that the growth at certain levels of diaminopurine, when present with adenine or guanine, was greater than that attributable to the adenine or guanine alone.

L. casei is one of those species in which there is extensive interconversion of the two purines. In folic acid deficient media, adenine can supply all of the adenine and guanine requirements, and similarly guanine can be converted into adenine and can supply all of the purine requirement. When both purines are available, the interconversion continues unabated and nearly half the guanine is converted into adenine and vice versa. The results of some four-score tracer experiments, selected ones of which are summarized in figure V, now confirm the assumption that the growth stimulation by diaminopurine is accompanied by its conversion into both adenine and guanine, and that under conditions where good growth is possible the three purines are essentially equally effective precursors of either of the PNA purines. In this figure, the pairs of columns represent the adenine and the guanine of the PNA, and the shading and arrows indicate the extent of incorporation and the direction of interconversion found for each added purine in the replicate experiments. The first through the third blocks indicate the essential

equality of these three purines as precursors of pentose nucleic acid purines. The last three demonstrate that 2,6-diaminopurine is still well-utilized when present at high levels and that the only obvious influence of large amounts of 2,6-diaminopurine is some reduction in the extent to which adenine is converted into guanine and vice versa. In these experiments, the somewhat preferential conversion of diaminopurine into guanine parallels the fate of guanine, and there is no suggestion that diaminopurine is a specific antimetabolite of adenine, at least in so far as the anabolism of pentose nucleic acids is concerned.

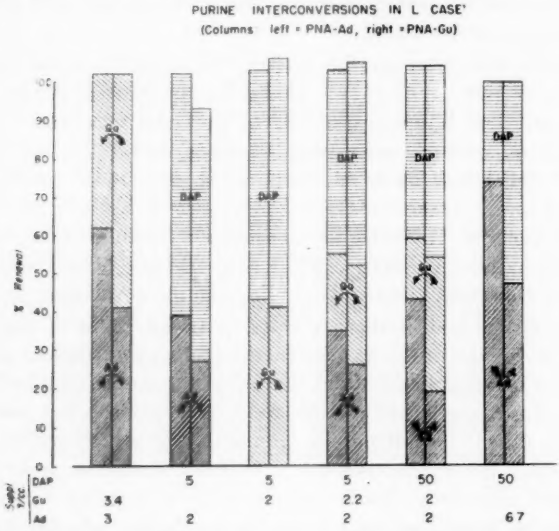


FIG. V. The incorporation and interconversion of adenine, guanine and diaminopurine by *L. casei*.

It was found that when the addition of diaminopurine is delayed for several hours, during which time the organism has been given an opportunity to acquire some adenine, either directly, by *de novo* synthesis, or by conversion of guanine, that diaminopurine is no longer inhibitory, all of the added sample can be used for nucleic acid purine synthesis. Thus, there seems to be some primary need for adenine with which diaminopurine can specifically compete.

In this respect, it is significant that a reverse situation also has been demonstrated (Dr. Robert Guthrie, unpublished). Certain purine requiring mutants of *Bacillus subtilis* can grow on diaminopurine or diaminopurine riboside but not on adenine or its derivatives, and the growth stimulation by diaminopurine can be blocked by the presence of adenine. The several observations that diaminopurine can serve as a metabolite and yet may be in competition with adenine are reminiscent of other instances where competition has been demonstrated between structurally related matabolites.

It is attractive to postulate that there is a primary requirement for adenine which is a reflection of a need for adenosine triphosphate (ATP) or for adenine containing coenzymes, and that it is here that diaminopurine is competing. That postulate gained credence from some studies by Doctor A. Kornberg who has been studying the specific adenosine-kinase which will phosphorylate adenosine in the 5′-position. We supplied eleven synthetic nucleosides, including five variants of the carbohydrate moiety of adenosine and six 2-substituted adenosines. The riboside of 2,6-diaminopurine was the only one of these nucleosides, besides adenosine, which proved (Kornberg and Pricer, 1951) to be phosphorylated by this enzyme. In the proper system, there can result a triphosphate derivative analogous in structure to APT, which can probably best be designated as 2-amino-ATP. Thus diaminopurine riboside is unique in being susceptible to two enzymes (adenosine deaminase and adenosine-kinase) which act upon adenosine, and those properties of diaminopurine riboside must be considered in any proffered explanation of the inhibitory effects of diaminopurine.

From the over-all results in *L. casei*, it can be interpreted that diaminopurine interferes with the early incorporation of adenine into some very essential product. Alternatively, it can equally well be interpreted that diaminopurine is incorporated into an analogous product by the same enzyme that is handling adenine. It is interesting to speculate that the products of that metabolism could conceivably be 2-amino adenine analogs of some dinucleotide coenzymes, which could be the ultimate "antimetabolites," possibly by disturbing normal balances of electron transport or other systems.

The author wishes to express his indebtedness not only to his associates in the Protein Division, but also for the cooperative efforts of Doctor C. C. Stock and the members of the Division of Chemotherapy and for the continued interest of Doctor C. P. Rhoads.

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INFLUENCE OF DIET ON SPONTANEOUS AND NUTRITIONALLY INDUCED TUMORS

R. W. ENGEL*

From time to time through the ages, diet has been thought to be responsible for all human ailments, including cancer. Many attempts have been made to correlate cancer incidence with dietary habits. The British Empire Cancer Campaign directed its efforts towards extensive surveys among various religious orders whose tenets in some cases forbade the eating of meats, or in other cases some other restricting or limiting regime. None of these studies have revealed any striking differences in cancer incidence among adherents of various diets as against the general population (Reimann, 1947).

The high incidence of primary liver carcinoma among the South African Negroes and the absence of any similar predominance of this type of tumor in the Negroes in the United States (Kennaway, 1944) suggests that some environmental factor, possibly diet, is involved. There may be extrinsic factors, other than diet, however, that have a bearing on the high incidence of primary liver cancer in the native South African.

The observation by Copeland and Salmon (1946) that insult to the liver in rats accomplished by feeding a diet deficient in choline would eventually lead to neoplasms in the liver and other tissues was, in this connection, a very significant observation. This observation, the first clear cut demonstration of a relationship between diet and spontaneous tumors in experimental animals, has restimulated a great interest in the overall problem of the genesis of cancer and the role of dietary factors in this process.

In this brief discussion, I will attempt to summarize the progress that has been made in the general field of relating tumor induction to a dietary deficiency of choline, or labile methyl supply.

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The diet used originally by Copeland and Salmon is given in Table 1. In this diet, referred to as the peanut meal-casein diet, about one-third of the protein is supplied by casein and about two-thirds by peanut meal. The latter is a protein extremely deficient in methionine. Consequently, the diet was deficient in labile methyl if no choline was supplied. The diet, however, was adequate in methionine when it was supplemented with choline. The vitamin supplement was designed to take care of the requirement of the rat with a relatively broad margin of safety. Animals that served as controls were given this same diet supplemented with two grams of choline chloride per kilogram of diet.

TABLE 1

Composition of Diet 43 A

Ingredient	Grams per kilogram
Alcohol—Extracted Peanut Meal	300
Alcohol—Extracted Casein	60
Sucrose	400
Lard	200
Salts 186	40

Each kilogram was supplemented with the following vitamins, in milligrams: thiamine and pyridoxine, 2 each; riboflavin, 4; calcium pantothenate, 10; i-inositol, 200; alpha-tocopherol, 50; beta-carotene, 4; and calciferol, 0.125.

This diet has been modified slightly from time to time without any apparent influence upon its tumor-inducing properties. Such changes were the inclusion of vitamin K, substitution of the carotene, and calciferol supplement with cod liver oil (one per cent), or reduction of the peanut meal component to 25 per cent.

This diet, without choline, when fed to weanling rats of the Alabama Experiment Station (AES) strain, will result in a 100 per cent mortality from an acute choline deficiency hemorrhagic dyndrome during the first two weeks. To insure against such fatalities, it is necessary to supply the experimental animals with minimal choline levels for the first ten weeks. Now this is done by initially feeding a 0.10 per cent level of choline chloride in the diet and reducing this level by 20 per cent every two weeks so that the choline supplement is eliminated after ten weeks. With this procedure, very few animals are lost with

acute choline deficiency symptoms. In some of the earlier work, this choline supplementation during the critical rapid growth period was accomplished in other ways, namely, subcutaneous injection several times daily of minimal levels of choline or intermittent stomach tube feeding of similar low levels. The first procedure described, namely the gradual reduction of choline mixed into the diet, was adopted because of convenience, and also it appeared to insure uniformly better survival.

The extraction of both the casein and the peanut meal with methyl or ethyl alcohol is important to insure that these materials are rendered as free of choline as is possible. The treatment employed usually reduces the choline content of the peanut meal from an initial level of about 2.5 mg. per gram to a level of 0.10 mg. per gm. or less.

A summary of the results obtained with this diet as reported by Copeland and Salmon (1946) is presented in Table 2.

TABLE 2

Incidence and Variety of Tumors in Chronic Choline deficiency in Rats
(Peanut Meal-Casein Diet)

Diet	Liver Cirrhosis	Liver Tumor	Other Tumors
Basal	50/50*	20/50	8/50
Basal + 0.2% of Choline Cl	0/19	0/19	0/19

*No. of animals affected/total no. of animals.

In this study, 50 animals were included in the report as having survived the early or acute choline deficiency syndrome and having been on the experimental diet for periods of eight to 16 months. All of these had liver cirrhosis at the time of death, and 20 of the 50 had liver tumors, either of the hepatoma type or the adenocarcinomatous lesion. In eight of the 50 animals, other types of neoplasms were noted. These were either fibrosarcomas or endotheliomas. The overall tumor incidence reported in this study was 58 per cent.

One of the questions that immediately might be asked is whether or not the peanut meal contains some factor or factors that might be conducive to tumor induction and whether or not the foregoing results would be obtained with another type of diet. Salmon had made the observation (1947) that when a

nine per cent casein-cystine supplemented diet contained 40 per cent of corn grits in place of sucrose, excellent growth resulted in young rats. It had been shown earlier in our laboratory (Engle, 1943) that corn products and particularly degerminated products were quite low in choline, containing only 0.10 mg. of choline per gram. Consequently, a diet of the type illustrated by diet 46 in Table 3 was selected. This was fortified

TABLE 3

*Composition of Corn-Grits-Casein Diets**

Ingredient	Grams per kilogram Diet No.		
	45	*46*	*46E*
Degerminated Corn Grits	400	400	400
Alcohol—Extracted Casein	90	90	45
L-Cystine	3	3	3
Sucrose	467	317	362
Lard	0	150	150
Salts	40	40	40

*The diets were supplemented with vitamins as indicated in Table 1.

with the usual B vitamins except choline. Diet 46E is identical with 46 except that the casein has been reduced from nine per cent to 4.5 per cent. This diet was not fed until the animals had been on diet 46 for six months. The summarized results (Engle, Copeland, and Salmon, 1947) are given in Table 4. The inci-

TABLE 4

Neoplasm Incidence in Rats Fed Corn-Grits-Casein Diets Deficient in Choline

Diet No.	No. of Animals	No. with Neoplasms	No. with liver Cirrhosis	No. with liver Neoplasms
45	8	0	0	0
46	14	6	6	0
46E	11	6	9	4

dence of liver cirrhosis was high on diet 46E (the low-casein diet); nine of the eleven animals were affected. Four of these eleven had liver tumors. On Diet 46 (the higher-casein diet) the incidence of liver cirrhosis was considerably reduced, being present in only six of 14 rats; and no liver tumors were observed. An observation that perhaps merits more attention is the relatively high incidence of tumors in sites other than the liver

that were observed with Diet 46. These included three sarcomas, two hemangiomas and one mammary tumor. Diet 45 is identical with 46 except that the 15 per cent lard in the latter has been replaced by sucrose. This low-fat diet appeared to afford complete protection against liver damage, and no tumors were observed.

The results, so far as liver tumor induction is concerned, looked promising with respect to diet 46E. However, a complicating factor that will be discussed later was encountered in these animals, namely, marked edema; this developed in about half of the animals, which usually reduced the experimental period through early death.

In view of the reported protective effect of riboflavin against liver tumor induction with azo dyes (Kensler, *et al.*, 1941), the reported procarcinogenic effect of pyridoxine (Miner, *et al.*, 1943), and the known lipotropic effect of inositol (Gavin and McHenry, 1941; and Engel, 1942), the results of a study illustrating the effects of these nutrients in chronic choline deficiency are given in Table 5. This is a summary of the data reported by Schaefer, Copeland, Salmon, and Hale (1950).

TABLE 5

*Incidence and Variety of Tumors Under Varying B Vitamin Intakes and Chronic Choline Deficiency in Rats**
(Peanut Meal-Casein Diet)

Diet Variation	Liver Cirrhosis	Liver Tumors	Other Tumors
Low-RF (1 or 2 mg./kilo diet)	11/12*	5/12	1/12
Varying Vit. B_6 (0.0.5, 1 or 10 mg./kilo diet)	30/34	14/34	3/34
Varying Inositol (0 or 200 mg./kilo diet)	8/8	4/8	0/8
Totals	49/54	23/54	4/54
Control Diets (0.2% Choline)	0/24	0/24	0/24

*No. of animals affected/Total no. of animals.

When the dietary riboflavin was lowered from the usual level of four mg. per kilo to one or two mg. per kilo, eleven of twelve animals developed cirrhosis, five had liver tumors, and one animal had sarcoma. Varying the pyridoxine in the diet from the

usual level of two mg. per kilo to the levels indicated, namely, 0 to 20 mg. per kilo, had no influence on the tumor incidence; therefore, the restuls have been combined. Fourteen of 34 rats in this series had liver tumors while three rats had respectively a sarcoma, a mammary tumor, and a tumor in the thoracic region. Omitting the inositol supplement failed to influence the results so that control and 0 level are reported together with four of eight animals having liver tumors. In all of these animals, the liver cirrhosis incidence was between 90 and 100 per cent. The control animals receiving choline were normal.

A result of particular interest in this study was the apparent complete protection against tumor development by high levels of riboflavin as shown in Table 6. The results with the low-riboflavin levels are shown here for comparison. Five of the twelve animals on the low-riboflavin diet had liver tumors, whereas no tumors were observed in the eight animals on the high-riboflavin diet (20 mg. per kilo). Although six of the eight animals on high-riboflavin diets had liver cirrhosis, it was much less severe than was observed with the low-riboflavin diets.

TABLE 6

*Influence of High Dietary Riboflavin on Tumors in Chronic Choline Deficiency in Rats**

Diet Variation	Liver Cirrhosis	Liver Tumors	Other Tumors
Low-RF			
1 or 2 mg./kilo diet	11/12*	5/12	1/12
High-RF			
20 mg./kilo diet	6/8	0/8	0/8

*No. of animals affected/Total no. of animals.

One of the questions of considerable interest was whether or not the tumors induced in rats under a chronic choline deficiency condition could be demonstrated in other species. To gain some information on this question, studies were extended to chickens and dogs.

Table 7 illustrates the summary data obtained with chickens as reported by Schaefer, Copeland and Salmon (1949). The diet used was the peanut meal-casein diet with slight modifications in the mineral and B vitamins components to bring them in

line with accepted standards in poultry nutrition. These chickens were initially fed the diet supplemented with either 0.10 or 0.30 per cent of choline chloride, and the choline was then gradually reduced to levels of .05 per cent or less. In 16 to 19 weeks the birds had been reduced to the low-choline level. The experimental period indicated is the period actually on the choline-deficient ration.

TABLE 7

Incidence and Variety of Tumors in Chronic Choline Deficiency in Chickens
(Peanut Meal-Casein Diet)
Experimental Period 14–44 wks.

No. of Chicks	No. with Tumors	Types of Tumors
23	12	3 Liver Adenocarcinoma 2 Intestinal Tumors 1 Hemangioendothelioma (Metastasis to Ovary) 1 Liver Cholangioma 3 Kidney Tumors 4 Fibrosarcoma 1 Benign Fibroma

Of the 23 birds that had expired at the time this report was made, twelve had tumors of one type or other, and the types of tumors noted are indicated. It appears that in the chicken there seems to be a greater variety of tumors produced in sites other than the liver. Four of the 23 birds had liver tumors whereas eight tumors of other types were identified. Of particular interest were the lesions in the kidney. Tumors in this organ have thus far not been observed in rats under these conditions.

The studies with dogs have been of unusual interest. The initial experiment with dogs is summarized in Table 8 from

TABLE 8

Influence of Prolonged Feeding of Choline-Low Diets to Dogs
(Peanut Meal-Casein Diet)
Experimental Period of 13 to 55 wks.

No. of Dogs	No. with Liver Cirrhosis	No. with Edema	No. with Duodenal Ulcers
7	6	6	6

the data reported by Schaefer, Copeland, and Salmon (1951). Here again, it was discovered early that low levels of choline had to be supplied to insure survival. Consequently, the survival period indicated is the average survival of the dogs while on a choline level of .05 per cent. Some of the dogs received higher levels of choline initially and were gradually depleted. The significant result in this study is the high incidence of duodenal ulcers. Perforated ulcers were the direct cause of death in two cases. Another complicating factor was the development of extensive edema, which presumably hastened the onset of death. Of the seven dogs that died in this initial experiment, six had duodenal ulcers, edema, and liver cirrhosis. The extent of liver damage was well-correlated with the survival period. The dog that survived only 13 weeks on the low level of choline died with a perforated duodenal ulcer, and liver cirrhosis was mild. Survival of 30 weeks or more on the deficient diet usually resulted in extensive nodular lesions in the liver. Some of these were described as hyperplastic regeneration nodules. Two-tenths per cent of dietary choline or the low level of choline and a source of vitamin B_{12} (Mercks Vit. B_{12} conc.) were effective in protecting the liver in control dogs and maintaining normal hemoglobin levels. The unusually high incidence of duodenal ulcers may or may not be directly related to choline. It is possible that inanition is a contributing factor in the development of this lesion since dogs on the deficient diet are quite variable in appetite behavior.

The production of liver tumors as a result of chronic choline deficiency in rats has been reported by other investigators. Data reported by Staub, Viollier, and Werthemann (1948) are summarized in Table 9. The composition of the diet was not given. The diet was reported to be choline-free and contain as the protein source a vitamin-free casein that had been treated with

TABLE 9

Pathology in Rats Fed Diets Containing Extracted Casein
Experimental Period 3–4 Months

No. of Rats	No. Dead with Kidney Hemorrhage	No. Dead with Lung Infection	Effective No. of Rats	No. with Liver Adenoma
106	20	17	69	10

.02 N. acetic acid under toluol, washed with water, dried, and extracted with 95 per cent alcohol for 48 hours. This whole process was again repeated and the product finally oven-dried for several days.

Of the 106 rats initially in the experiment, 20 died with kidney hemorrhage; an additional 17 were lost with what was described as a chronic lung infection, leaving a total of 69 effective animals in the study. Of these, ten were reported as having liver adenoma.

Best (1950) reported before the joint session of the Federation of American Societies for Experimental Biology in 1950 that with the Wistar strain of rats a few tumors had been noted in animals that had been kept on a diet low in lipotropic factors for as long as 16 months.

Erickson and Goebbel (1949) of the Duke University reported before The American Society for Experimental Pathology at the Federation meetings in 1949 that, although tumors were obtained in their rats of the Osborne and Mendel Strain, the high tumor incidence reported from the Alabama laboratory was not observed. These workers used a diet identical with the diet used by the Alabama workers.

Viollier (1950) has recently reported a series of studies in which the activity of several enzymes was compared in the livers of rats bearing tumors produced either by a deficiency of choline or the feeding of azo dye. The number of rats involved in the study was not indicated. Liver lesions, described as adenomas, were produced in 20 to 50 weeks by feeding a diet deficient in choline. The details of the diet were not given.

Confirmatory evidence of the induction of tumors through a prolonged dietary deficiency of choline has thus been obtained in several other laboratories. Some of these reports indicate less striking results than those reported in our laboratory. There may be several explanations for this and I would like to advance at least a few possible explanations. First of all, the thorough extraction of the peanut meal is of particular importance since this ingredient is a rather good source of choline when compared with other plant products. Extraction would also aid in rendering the diet low in vitamin B_{12} and folic acid, factors which are known to decrease the dietary requirement for choline (Schae-

fer, Salmon, and Strength, 1949). The strain of rats used also may have an important bearing on the results.

Chronic choline-deficiency studies have thus far been conducted only with the Alabama Experiment Station strain of rats in our laboratory. Data obtained with this strain and the Wistar strain in acute choline deficiency studies are given in Table 10. These are summary data of the results reported by

TABLE 10

Strain Differences in Choline Requirement in Rats

Diet	Strain of Rat	No. of Rats	Kidney Hemorrhage Incidence %	Fatal Kidney Hemorrhage Incidence %	
31 PMC* + .04% Choline	Wistar	60 M	48	10	F_1 Generation Rats
		66 F	20	2	
	AES†	43 M	100	60	
		33 F	94	43	
31 PMC + .03% Choline	Wistar	14 M	50	14	
		18 F	22	0	
31 PMC + .06% Choline	AES	15 M	100	67	
		19 F	84	36	

*Same as Diet 43A except that the lard is reduced to 6 per cent.
†Alabama Experiment Station Strain.

Engel (1943) and Copeland (1946). The diet used here is the same as the peanut meal-casein diet routinely used in the chronic studies except that the lard in the diet is at a six per cent level rather than at 19 per cent, with a compensatory change in dietary sucrose level, weight for weight.

F_1 generation weanling rats from both the AES and the Wistar strains were fed the basal diet supplemented with a low level of choline (.04 per cent). The incidence of choline deficiency kidney hemorrhage was twice as high in males of the AES strain as in males of the Wistar strain. The differences between the females of the two strains appeared even more exaggerated. Likewise, the fatal kidney hemorrhage in the AES strain was much higher than in the Wistar strain. Weanlings in the F_2 generation were used to get at the quantitative difference in choline requirement between the two strains. Rats of the Wistar strain were better protected against the acute choline-deficiency hemorrhagic syndrome when the diet was supplemented with

.03 per cent of choline than were AES rats receiving the dietary supplement of twice that level, namely, .06 per cent choline.

More recently, differences in behavior of the rats of the Sprague-Dawley strain and those of the AES strain have been studied (Hale and Schaefer, 1951). Results obtained with a diet that was developed in our laboratory for the production of nutritional edema (Engel and Alexander, 1951) are given in Table 11. The diet used is similar to peanut meal-casein diet used in chronic studies except that both protein components have been reduced, the casein from the normal level of six per cent to two per cent and the peanut meal from a normal level of 30 per cent to ten per cent. Sixteen of 29 rats of the AES strain died with acute choline-deficiency kidney hemorrhage whereas only five of the 101 rats of the Sprague-Dawley strain died from this cause (Engel and Alexander). These results indicate that there is a difference between these two strains with respect to the choline requirement although the differences in requirement do not appear to be as great as the differences noted between the Wistar strain and the AES strain.

TABLE 11

Incidence of Fatal Choline Deficiency Renal Hemorrhage in Rats of 2 Strains Fed Diet C-189

Strain*	No. of Rats	No. with Fatal Kidney Hemorrhage
AES	29	16
S. D.	101	5

*AES—Ala. Exp. Station Strain. SD—Sprague Dawley Strain.

Interestingly enough, these differences in strain behavior are also evident when liver-tumor-inducing azo dye was fed (Engel, 1952). Weanling rats of both the AES and the Sprague-Dawley strains were fed the peanut meal diet supplemented with choline and with .045 per cent of p-dimethylaminoazobenzene. After four months, the animals were sacrificed, and the gross condition of the livers in the two strains was compared. Livers of the AES strain appeared to be strikingly more susceptible to damage from the azo dye than did livers of the Sprague-Dawley strain. Five of the ten livers from the AES

strain had well-defined tumor nodules. None were noted in the livers of the Sprague-Dawley strain. Furthermore, the livers of the AES rats in general exhibited rather severe cirrhosis and scarring whereas those of the Sprague-Dawley strain were nearly normal in gross appearance.

It should be emphasized at this point that from these results it should not be concluded that other investigators studying chronic choline deficiency in the rat have obtained less striking results with respect to tumor production than the results reported from the Alabama laboratory simply because they were dealing with rats of different strains. However, the results do suggest that the strain of rat used may have an important bearing upon the results.

Summary

The initial observation that the prolonged feeding of diets deficient in choline promotes the production of tumors in the liver and other sites in rats has been amply confirmed by additional studies in our laboratory as well as by workers in several other laboratories. This promotion of spontaneous tumor growth through dietary restriction of choline has also been demonstrated in chickens. No tumors have been observed in dogs in the presently reported studies. The complication of early edema development was also noted to occur in rats when attempts were made to hasten the damaging action of choline-low diets by reducing the dietary protein.

It has been pointed out that strain differences in rats may have an important bearing on the results in choline deficiency studies and that at least two strains exhibit markedly different susceptibilities to liver tumor induction by azo dyes. Early results indicate that the striking anticarcinogen action of riboflavin in azo dye carcinogenesis has its parallel in the action of riboflavin in preventing liver tumor induction by the feeding of a low-choline diet.

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HEREDITARY INFLUENCES IN MELANOMA DEVELOPMENT*

MYRON GORDON

Fundamental discoveries in the biological studies of pigment cells with regard to their origin, growth, and heredity were made independently of, and prior to, their application towards the understanding of the nature of the melanoma in man.

Embryologists working with the lower vertebrate animals proved experimentally that pigment cells, no matter where they were found in the adult, arose chiefly from the neural crest. First discovered from work with the salamander, biologists later found similar proofs of origin of the pigment cells from neural crest cells in the bird, mammal, and fish. From this evidence, it is most probable that the pigment cells of man have a comparable origin. These biological discoveries have clarified the multiple and contradictory theories that were held concerning the embryonic origin of those tumors that arise from the atypical growth of pigment cells.

At the Third Conference on the Biology of Normal and Atypical Pigment Cell Growth, a spontaneous discussion arose on the subject of nomenclature. Some medical workers were inclined to use the term *melanophore* (or *chromatophore*) to denote any pigment-bearing cell. Some used it to denote a cell that obtains its coloring matter by phagocytizing particles released by those pigment cells which are capable of synthesizing their contained pigment. Biologists pointed out that the word *chromatophore*, though often used to indicate any animal pigment cell, is more appropriately employed for those specific pigment cells which have certain unique physiological properties. The pigmented matter in chromatophores may be dispersed throughout the cell or concentrated in a small area in the center of the cell, depending upon the physiological state of the organism. Animals having this type of pigment cell are able to change their color to match their environment. The term chromatophore was first

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used in this sense. according to P. H. Parker (1948) in the Italian form *cromoforo* by Sangiovanni, in 1819. Chromatophores are generally classified in accordance with either the chemical nature or appearance of the coloring matter which they produce and carry. Biologists have used the term *melanophore* for the cell that produces dark brown or black pigment (melanins) and has the physiological properties mentioned above. The members of the Conference suggested that all workers in pigment cell biology restrict the use of this term to its original biological meaning. It was recommended that the term *macrophage* (not melanophore) be used to denote a phagocytic cell containing ingested pigment.

Two further suggestions were agreed upon at the Conference: (1) that the term *melanocyte* be used for the basic pigment cell that is capable of synthesizing melanin and contains this pigment, but does not have the physiological properties of the melanophore, and, (2) that the term *melanoblast* be used to refer to the embryonic, non-pigmented precursor of the melanocyte.

This clarification of nomenclature is desirable, particularly so for the presentation which follows because the proof for the hereditary influence in melanoma development rests upon the activity of large melanophores (or *macromelanophores*) in members of different genetic populations of fishes. Since a detailed review of the work on the genetic and correlated studies of normal and atypical pigment cell growth in fishes will soon be published by Gordon (1952), the following is but a summary of the material that will be presented in a series of lantern slides at this meeting.

Almost a quarter of a century ago, proof was available that macromelanophores in the Mexican Flatfish, *Xiphophorus (Platypoecilus) maculatus* were inherited. The experiments indicated these large black pigment cells are transmitted to their descendants by means of dominant genes, five of which form a series of sex-linked alleles. In the members of three natural populations of Mexican platyfish, the type of sex linkage may be expressed by the formula XX equals femaleness, XY equals maleness; or in other words, the female is the homozygous sex, while the male is the heterozygous one, somewhat like that

in man. In the members of the natural population of platyfish from the Belize River in British Honduras, the opposite type of genetic mechanism for sex determination is found, namely, the male represents the homozygous sex while the female is the heterozygous one. This interesting but complex situation should not obscure the more important fact that macromelanophores are inherited in a known and predictable manner.

Every wild population of platyfish collected and analyzed indicates that macromelanophore patterns are found in all groups. Furthermore, all wild platyfish that carry macromelanophore patterns are normal with respect to the growth of these pigment cells. Not a single wild caught platyfish was found in over 10,000 specimens collected that had any indication of melanosis. Yet experiment after experiment in this laboratory and in several laboratories in Europe reveals this point: if a normal macromelanophore-carrying platyfish is mated with a normal swordtail (a related species, *Xiphophorus hellerii*) those of their hybrids that receive the macromelanophores develop either melanosis or melanoma. Those that do not receive the large black pigment cells in inheritance are normal.

Macromelanophores appear much earlier in the spotted platyfish-swordtail hybrids than in their spotted platyfish parent. The large black pigment cells first multiply in the corial tissues and then invade the subcutaneous regions of the hybrid. They are capable of destroying muscle and bone tissues. Eventually, in some individuals, a large tumorous mass is formed that is clearly visible externally. In the last stages of the disease, the tumor mass, when examined from cross-sections, is composed of relatively few macromelanophores and many melanocytes. When pieces of the melanoma are tissue-cultured, the cells found are predominantly dendritic melanocytes. These bear a striking resemblance to the dendritic melanocytes obtained from tissue-cultured human and mouse melanomas. This observation, united with the finding that pigment cells of all classes of vertebrate animals arise from the neural crest, is the basis for the thought that biological discoveries concerning the nature and behavior of pigment cells in any vertebrate animal are of great potential value to the understanding of the causes of abnormal growth of pigment cells in man.

The hereditary studies of abnormal pigment cell growth in fishes have indicated that in many instances the site of melanoma development in the hybrid may be predicted when animals of known genetic constitution are mated. One macromelanophore gene (with certain modifying genes) will cause the hybrid animal to develop a melanoma on the dorsal side of the body, while another will cause it to form a melanotic tumor along the ventral areas; still other genetic combinations will create conditions in the hybrid in which the melanoma will appear on its flanks.

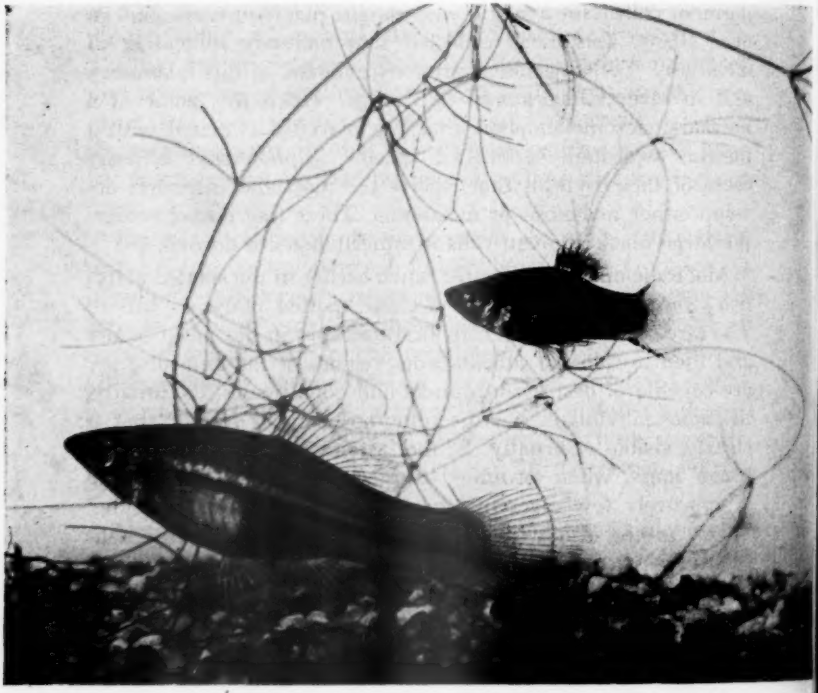

FIG. I. Normal parents: The male platyfish (above) has a hereditary black mark on its dorsal fin consisting of small tight cluster of macromelanophores. The female swordtail (below) is a closely related species. For some of their hybrids see figure II.

The unpigmented, or amelanotic melanomas, have been a paradox for some time. Experiments with genetically known and pure albino fishes have revealed that within limits, the presence of absence of melanin pigment in melanomas is a reversible proccess that is genetically controlled. Geneticists were able to show that when *albino* gene is present in a homozygous recessive state in conjunction with a macromelanophore gene and its modifiers, an amelanotic melanoma may be produced in a hybrid animal. An animal so produced when mated back to a normal black-eyed, golden swordtail will produce offspring, some of which have melanomas that, again, are black.

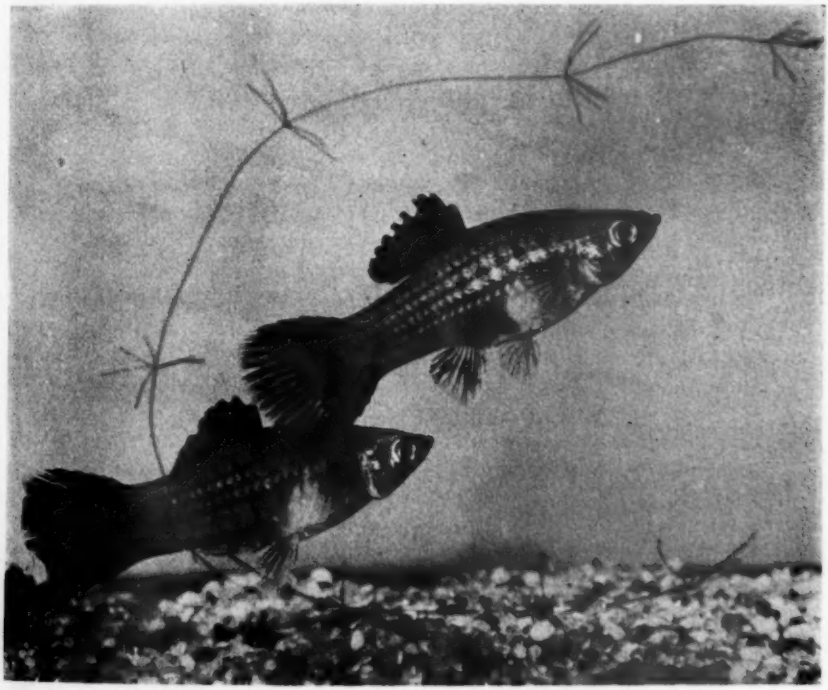

FIG. II. Hybrids with melanotic tumors of the dorsal fin. The upper fish is a female, the lower a male. Note the destruction of part of the fin indicating a state of melanosis initiated by the abnormal growth of macromelanophores.

Pigment cell growth was altered by genetic methods in hybrids of two closely related species of swordtails. A member of one species had a small macromelanophore pattern at the base of the tail; the other did not. The hybrids that inherited the gene for this tail-spotting developed melanomas. It became apparent that the side of the melanomas in these hybrids could be predicted for it always was initiated in the caudal region of

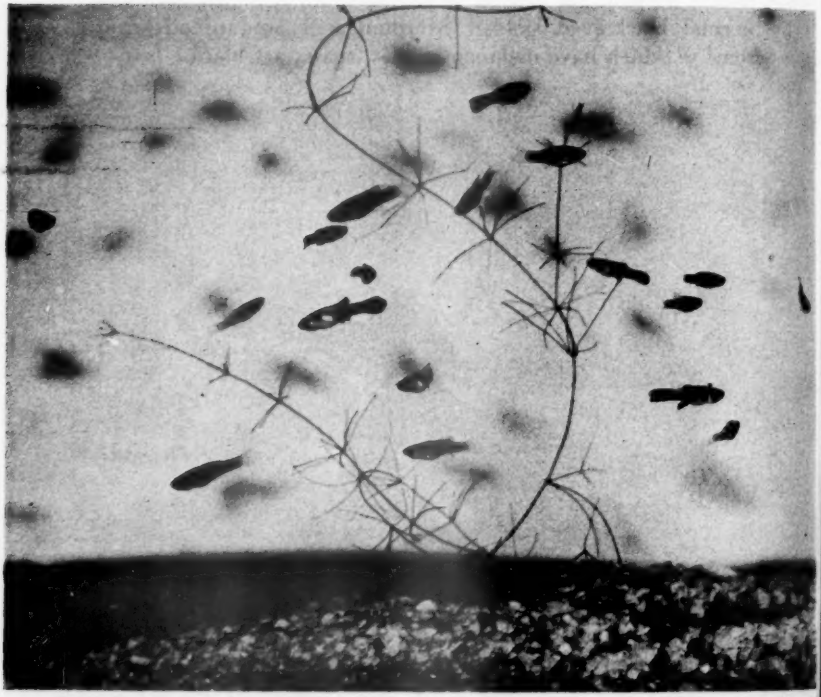

FIG. III. Platyfish-swordtail hybrid siblings one month old. Approximately half of them inherited macromelanophores—half of them did not. Those that did show a state of melanosis, an atypical growth of macromelanophores which usually precedes the development of melanomas. All these fishes had the same mother in which they developed over a gestation period of 30 days. They were all born on the same day and grew in the same aquarium water. The environment for all was identical, yet the 50 per cent which had macromelanophores also showed atypical pigment cell growth, while the 50 per cent with micromelanophores only were normal.

the body and spread anteriorly. Eventually, the entire body was blackened in some hybrids—but the melanoma proper usually appeared at the site of the initial macromelanophore pattern. This melanoma was transplantable, but the transplant grew very slowly.

No metastases were detected in any of the fishes with melanomas genetically induced. While metastases have been reported in some other tumorous fishes, they are quite rare, and this has been attributed to certain anatomical and physiological properties of fishes.

In the initial stages of melanoma development, the atypical growth of pigment cells in man and in fishes are quite similar. This has been evaluated by comparing the history of melanoma development in man, as painstakingly described by Dawson (1925), with the progressive stages in the growth of similar tumors in fishes.

In order to draw a closer parallel of the hereditary influence in the development of melanomas in fishes with some of the possible genetic influences in the etiology of human cancers, a new series of experiments was begun a few years ago and is still in progress. Objection may be validly drawn to any comparison of abnormal growth of cells which depend upon the combination of hereditary factors originating in two dissimilar species. The main body of information from genetic work on fishes was obtained from the crossing of members representing different species. Obviously, man does not reproduce by this type of mating. Most biologists and anthropologists agree that all races of mankind represent divergent groups of the same species.

From a number of collecting expeditions to Mexico, Guatemala, and British Honduras, some wild platyfish were obtained from three different and geographically isolated rivers. These geographical groups of platyfish represent a single species, since all have similar morphological traits. Genetic differences between them have been found—the chief difference being that each population had a distinctive frequency of pigmentary patterns. These differences were somewhat like the varying frequencies determined for the various blood types in geographical, isolated populations of man.

In matings between members representing different river populations of the platyfish, some of the macromelanophore-carrying intraspecies hybrids developed conditions of severe melanosis. This abnormal growth of macromelanophores paralleled that seen in platyfish-swordtail hybrids. In the latter, melanosis generally leads directly to the development of melanomas, but in the intraspecific platyfish hybrids, no melanomas have yet developed. From the preliminary experiments, it may be said that the early stages of atypical growth of macromelanophores are about the same in both types of hybrids and are fundamentally the same.

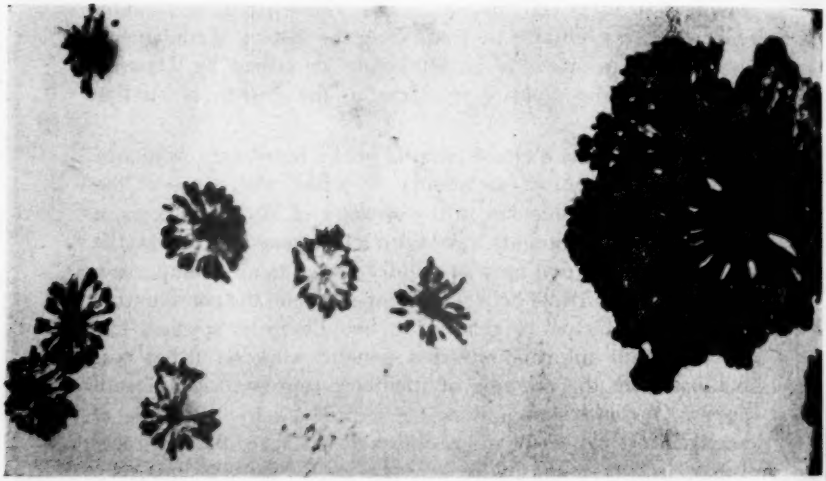

FIG. IV. The macromelanophore (to the left), the large black pigment cells that initiate the development of melanosis and melanomas in genetically imbalanced fish hybrids, may be distinguished from micromelanophores (to the right). The latter are smaller and do not lead to abnormal pigment cell growth. Melanophores are specialized pigment cells that are capable of spreading their contained pigment to the periphery of the cell or withdrawing it to the center of the cell. Melanophores are not macrophages, for they are capable of synthesizing melanin. (Photo from unstained skin of a normal platyfish).

Our present results from all genetic experiments designed to evaluate the hereditary influence on normal and atypical pigment cell growth may be explained within the framework of

present-day principles of Mendelian inheritance. The hereditary control of melanomas in hybrid fishes involves not one gene but a constellation of interacting genes. To be effective, the assemblage of genes that influence the atypical growth of pigment cells must contain one gene for macromelanophores. These large black pigment cells are particularly sensitive to the influence of genetic imbalance.

NATURALLY OCCURRING DIETARY AGENTS AND THEIR ROLE IN PRODUCTION OF TUMORS

CORNELIA HOCH-LIGETI*

With the increasing knowledge of the occurrence of tumors among all human races, it became evident that although malignancy seems to occur with similar frequency among the various races, the localization of tumors is strikingly different. Well-known examples are the low incidence of breast carcinoma in the Japanese women (Hoffmann, 1915), the absence of carcinoma of the penis in the semitic races circumcised prior to the eighth day of life (Kennaway, 1935), and the low incidence of uterine carcinomas in Jewish women (Kennaway, 1948).

Carcinomas of the liver, a relatively rare occurrence in the white races, is prevalent in certain parts of Asia and Africa. The prevalence of liver tumors among the colored population of the Dutch Indies was first described by Snyder and Straub in 1921, that in Java by Bonne in 1935, and that in South Africa by Berman in 1935. Figure I, taken from Berman's paper, shows the anthropological distribution of liver tumors as percentage of carcinomas of all sites. Berman (1941) enumerates certain conditions which are found together with liver tumors such as malaria, heliminthiasis, schistosomiasis, hemochromatosis, and alcoholism; but none of these appears to be connected consistently with the occurrence of tumors in the liver. Bonne (1935) points out that helminthiasis is unknown among the Malays in Java and that they consume very little alcohol. The question of the racial susceptibility was investigated by Kennaway (1944) who analyzed the data on incidence of primary liver cancer among the negroes in America and Africa. Since the former are descendants of the West African negroes, their racial susceptibilities should be identical. The data available did not indicate that the incidence of primary liver cancer among American negroes differs from that of the white people in America. Accordingly, the very high incidence of these tumors in African negroes cannot be of purely racial character.

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Some extrinsic factor might be involved in the case of African negroes.

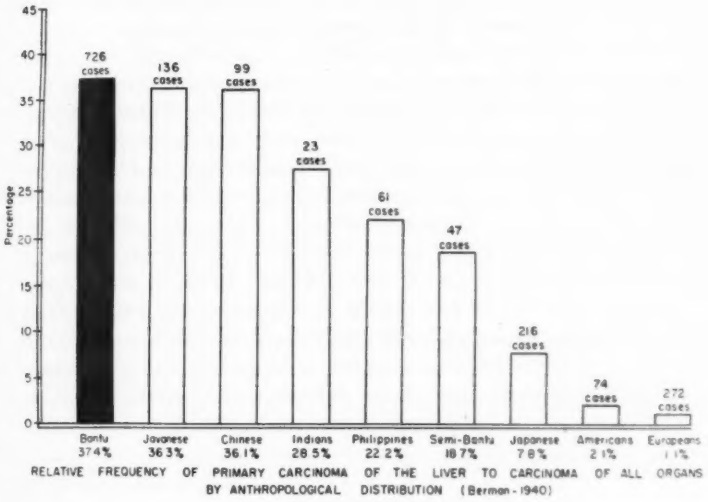

FIGURE I

The role of nutritional factors in the development of liver tumors is of great interest, particularly in view of the work on nutritional liver damage by György and Goldblatt (1939, 1940), Himsworth and Glynn (1944, 1944) and Daft *et al.* (1941). Emphasis was laid by these authors on the quantity of protein in the diet, its amino acid composition, particularly with regard to sulphur-containing amino acids (methionine and cystein), on the necessity for labile methyl group in the diet and on the different minimal requirements of vitamins in diets with different protein contents. Gilbert *et al.* (1943) and Gillman *et al.* (1945), demonstrated that maize-meal porridge, one of the staple foods of African negroes, induces liver damage when fed to young rats for a prolonged period.

We have approached the problem of nutritional factors in the development of liver tumors along two experimental lines.

"(a) Search for the presence of substances in the diet which might counteract the formation of hepatic tumors by other agents.

(b) Investigation of factors, present in food, the consumption of which might result in the formation of liver tumors.

With regard to the first possibility, it is well established that the activity of at least one hepatic carcinogen, p-dimethylaminoazobenzene (P.D.A.), is largely dependent on dietary factors. Yeast and liver, both rich in vitamins of the B complex, riboflavin, and protein, separately or in combination and hydrogenated coconut-oil were found effective in preventing tumor formation (reviewed by Burk and Winzler, 1944). In order to investigate the effect of a widely used food stuff, fresh milk was fed to rats receiving P.D.A. (Hoch-Ligeti, 1946) as carcinogen. Sugiura (1944) fed dried milk to rats receiving P.D.A. Both experiments showed that milk protected the liver; the effect of fresh milk, however, was superior to that of dried milk. Figure II shows that in the liver of rats fed a rice diet containing 0.07%

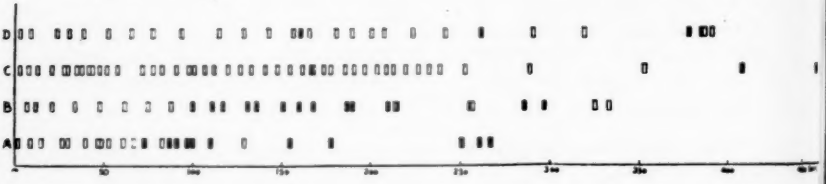

FIG. II. Incidence and time of occurrence of hepatic tumors in rats on different diets receiving p-dimethylaminoazobenzene.

A = rice and carrot diet.
B = rice, 6% casein, and carrot diet.
C = rice and milk diet.
D = rice and milk diet (second series).

Empty blocks represent rats without liver tumors: solid blocks represent rats with liver tumors.

P.D.A., tumors were found in all except of three animals killed after the 73rd day of the experiment. The addition of ten ml. of fresh milk daily largely prevented the development of hepatic tumors even in animals receiving the dye continuously for more than 15 months. Only six out of 80 rats developed tumors. In all livers with tumors, cirrhosis and/or cholangiofibrosis was

present. The free HCl in the stomach generally disappeared on diets producing tumors. Many rats developed papillomatosis of the stomach.

It is extremely difficult to collect data on the nutrition of primitive people; in particular, it is impossible to given an answer to the question whether any large proportion of them consume milk. Yet it is fairly certain that in regions of Africa where the tsetse fly is endemic, no cow can live, and that the natives have no milk, except possibly that from goats. In areas where milk is available, its consumption may be restricted because of economic causes or religious customs. It is stated that in some Bantu communities the women drink milk, but that it is avoided by males. In Japan, the consumption of milk, which was introduced to that country only at about the end of the 18th century, is negligible (Bourne, 1946).

Milk, however, did not have a general anti-carcinogenic effect. Rats in which the liver had been protected with fresh milk, were fed P.D.A. and developed pancreatic tumors. These tumors appeared in the 16th and 18th month of the experiment. Rats which did not receive milk already long succumbed to liver tumors (Hoch-Ligeti, 1949).

Although these experiments showed that dietary factors may be involved in the prevention of liver tumors due to carcinogenic agents, it remained to be shown whether any actively carcinogenic compound is consumed by a population with a high incidence of liver tumors.

Bonne (1935) in his paper on the incidence of hepatic tumors in the Malays and Chinese in Java and Sumatra writes: "The very strongly spiced food, especially of Malays, has been considered to contain carcinogenic irritants." He abandons this possibility because he assumes that such an irritant would first attack the wall of the stomach and intestine. The incidence of carcinoma of the stomach is, however, extremely low among the Malays but high among the Chinese.

One of the spices used extensively by the Javanese is chilli. In 1937 the International Research Foundation obtained a supply of chillies from Java through the good offices of the late Dr. Cornelius Bonne for investigations started under the direction of Professor E. L. Kennaway. The chillies from Java were the

species *Capsicum fruitescens* (Linn.) and *Capsicum annuum* (Linn.). The pungent compound of chillies, capsaicin, had been separated by Thresh (1876) and synthetised by Nelson and Dawson (1923). It is the vanillylamide of the Δ^6-8 methylnonenic acid and has the formula shown in figure III. Chillies contain about 0.1 per cent capsaicin.

CAPSAICIN

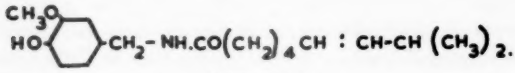

FIGURE III

In 1940 Dr. I. Hieger (personal communication) fed 30 mice a diet containing chillies and supplemented with milk. After 14 months, one hepatoma was found in one mouse. In 1948, it was reported (Hoch-Ligeti) that out of 30 rats which for about two years received chillies at a level of ten per cent in a semi-synthetic diet, seven developed changes in the liver resembling incipient hepatomas and cholangiomas. Malignant tumors were found in three rats. These rats were fed an adequate diet containing 17 per cent casein. Because of the late appearance of the tumors, the findings were considered inconclusive. It was thought that the combination of chillies with a diet devoid of all first class protein might accelerate the development of hepatic tumors. In a second experiment (Hoch-Ligeti, 1951), the casein in the food was therefore replaced by "ardein," a purified protein from the ground-nut. The main difference in the amino acid composition between ardein and casein is, as shown in figure IV, that ardein has a high percentage of arginine (13.1

FIGURE IV

Main Differences in the Amino-Acid Composition of Casein and of Ardein

	Casein (Block 1945)	Ardein (Chibnall 1948)
Arginine	4.1%	13.1%
Cystine	0.36%	1.58%
Methionine	3.5%	1.24%
Total S-containing amino-acids	3.86%	2.82%
Cystine / Methionine	0.1	1.28

per cent) and cystine (1.58 per cent) and a low methionine (1.24 per cent) content (Chibnall, 1948). The corresponding figures for casein are: arginine 4.1 per cent, cystine 0.36 per cent and methionine 3.5 per cent (Block and Bolling, 1945). Accordingly, the cystine methionine ratio is 0.1 for casein and 1.28 for ardein.

In the livers of all rats, irrespective of whether their diet contained chillies or not, the most prominent pathological feature was gross fatty changes. This change was most pronounced in the right lobe. In several livers, proliferation of the connective tissue occurred to a varying extent; in some a frank cirrhosis superceded the fatty changes. The deposition of ceroid was present in many instances.

In the livers of 26 rats which were fed with chillies and killed after six months, neoplastic changes were found in 15 cases. The tumors were cystic cholangiomas, solid cholangiomas, adenocarcinomas, and hepatomas. One hepatoma metastasized to the lymph nodes and to the omentum, figure V. No tumors of other localization were observed.

As the cystine-methionine ratio is very high in ardein, it is conceivable that the rats suffered from secondary choline deficiency. The addition of methionine, however, had no effect on the rats. Copeland and Salmon (1946) described the development of hepatic tumors in rats fed choline-deficient diets. In the experiment reported here, no tumors occurred in the rats receiving the basic diet only. Potter *et al.* (1942) have described extremely fatty livers in dogs with riboflavin deficiency. The symptoms of the rats succumbing in the earlier parts of this experiment, *e.g.*, spastic paralysis of the hind leg, loss of hair, were similar to those of riboflavin-deficiency.

In the next series of experiments (Hoch-Ligeti, unpublished data), excess vitamin B complex was given to rats fed on ardein-chilli diet as previously. One group, however, was kept choline-deficient, and another riboflavin-deficient. The experiment had to be terminated after nine months. While extremely fatty livers were found in the rats kept riboflavin-deficient, no cirrhosis was present in these livers, and no tumors developed. In the choline-deficient rats, the livers showed much cirrhosis, and 13 out of 30

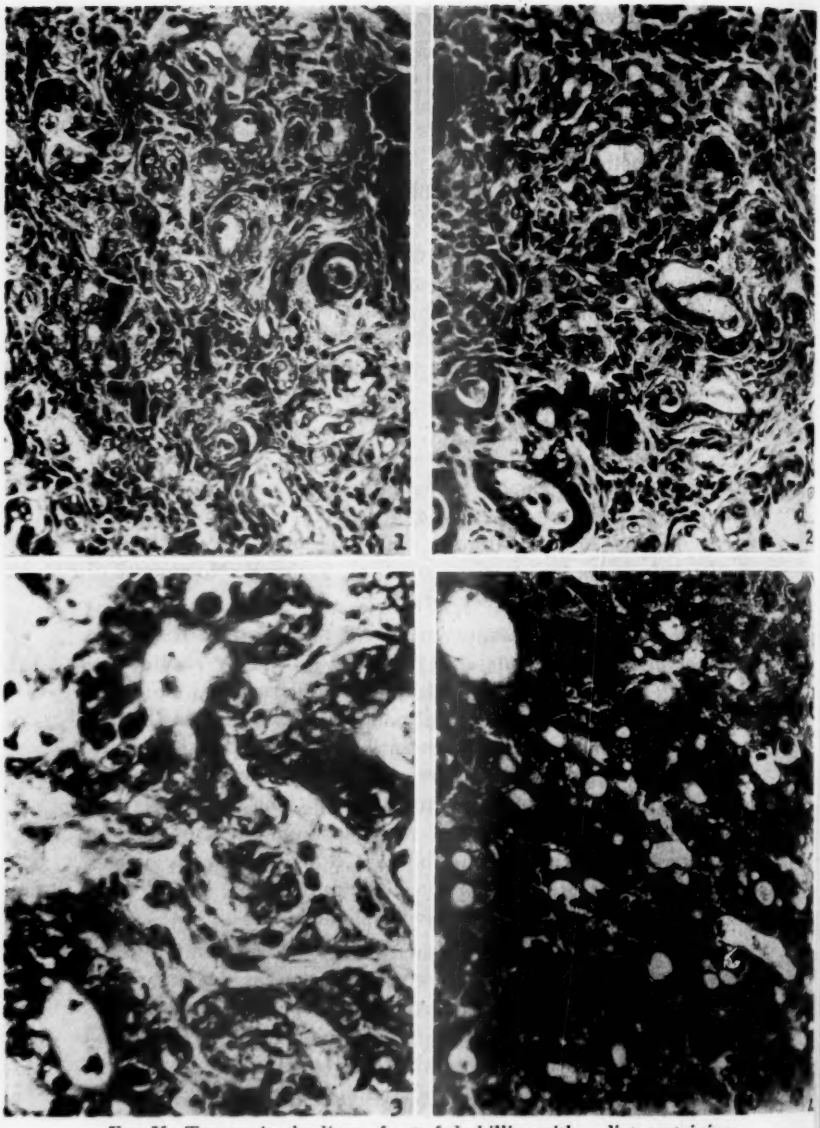

FIG. V. Tumors in the liver of rats fed chillies with a diet containing ground-nut protein.

Photomicrograms 1 and 2. Solid cholangiomas, x 185.
3. Adenocarcinoma, x 300.
4. Hepatocellular carcinoma, x 250.

These rats were killed between the 10th and 12th months of the experiment.

showed tumor-like formations, most of them cystic cholangiomas. One hepatoma and one adenocarcinoma were found. No tumor metastasized. Since this experiment had to be terminated at a rather early date, it is difficult to draw final conclusion from it, but choline did prevent cirrhosis and tumor formation though it did not prevent fatty changes on a riboflavin-deficient diet.

Along similar lines of reasoning, *i.e.*, that liver tumors might be produced by some extrinsic dietary factor, Cook *et al.* (1950), investigated the effect of feeding alkaloids of the Senecio plants to rats. These plants are used in certain parts of Asia and Africa as medicine, charm, stmulus, etc. In three out of eleven rats, liver tumors were found after three months of feeding the alkaloid with an adequate diet. Experiments will be carried out in our laboratory to investigate the effect of protein-deficient diets on the occurrence of these tumors.

The sequence of events seems to suggest that at least two factors are involved in the nutritional cancer production in the liver: first, a damage of the liver cells, which generally is not adequate for provoking tumors; and second, an agent which for the time being I can only describe as an irritant. This might be chilles, Senecio, or perhaps many other substances. Both chillies and Senecio are weak carcinogens when fed with an adequate diet. Accordingly, these substances seem to represent the more specific factor.

I should like to conclude by pointing out that the tumors produced in the livers of rats by the combinations of naturally occurring and widely used dietary factors with deficient diets rarely show histologically or clinically the degree of malignancy of the liver tumors frequently seen in humans. Although we were able to show the importance of nutritional habits both in the prevention of hepatic tumors due to a corcinogenic agent and in the production of hepatic tumors by purely dietary means, the correlation and integration of these findings into a pattern of carcinogenesis requires further investigation.

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RELATIONSHIP OF NUTRITIONAL FACTORS TO THE CHEMICAL INDUCTION OF TUMORS

CHARLES J. KENSLER*

Of the various molecular types of chemicals which have been found to produce cancer in one or more species of animals, the activity of only two groups of these compounds has so far been found to be appreciably altered by the composition, as distinct from the amount, of the diet fed. These are the azo dyes which are carcinogenic for liver and bladder and 2-amino-fluorene derivatives which produce tumors of the liver, mammary glands, and other tissues. Although both compounds produce cancer in several species of animals, almost all of the nutritional work has been done on the rat. In the present discussion, a brief summary of the nutritional factors which have been shown to influence tumor production by these agents will be presented and, insofar as possible, their mode of action considered.

The carcinogenic action of the azo compounds has been known for 20 years (Yoshida, 1922) and the fact that their carcinogenic activity can be grossly altered by diet was discovered in 1938 (Ando, 1938; Nakahara, Fujiwava, and Mori, 1939). This work and the major part of the later work has been done employing p-dimethylaminoazobenzene (DMB) as the azo carcinogen, although its action has been found to be more readily prevented by diet than that of closely related but more active compound, 3′ methyl-p-dimethylaminoazobenzene (3M-DMB) (Giese, *et al.*, 1946). The carcinogenic action of 2-acetyl aminofluorene (AAF) was discovered in 1941 (Wilson, DeEds, and Cox, 1941), and it has only recently been shown that diet can also influence the induction of tumors by this agent (Wilson and DeEds, 1950, Engel and Copeland, 1952). No specific compounds have as yet been implicated in the protection against AAF in contrast to the number of pure vitamins and other factors which have been shown to influence azo dye carcinogenesis.

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Three years after the discovery that crude liver and yeast supplements protected against DMB induced liver tumors in rats, this protective action was duplicated to a limited extent by the vitamin riboflavin (Kensler, *et al.*, 1941) when it was included in the ration in amounts larger than the usual nutritional requirement. This protection was voided in the absence of adequate protein intake (Kensler, *et al.*, 1941). Low protein intake also prevents the retention of riboflavin in the liver (Sarett and Perlzweig, 1943; Unna, *et al.*, 1944). Today, riboflavin is still the only pure vitamin supplement which has been found to reduce tumor incidence (Table 1). However, two other vita-

TABLE 1

Effect of Vitamin Content of the Diet on Liver Tumor Production by P-Dimethylaminoazobenzene

Vitamin	Tumor Incidence in deficiency	Tumor Incidence in excess
Riboflavin (1941)	increased	decreased
Pyridoxin (1943)	decreased	no effect
Biotin (1942)	decreased	increased
B_{12} (1950)	?	increased

mins have been reported to increase tumor incidence under certain nutritional conditions. These are biotin (du Vigneaud, *et al.*, 1942) which was found to decrease the protection afforded by the riboflavin-protein supplement and vitamin (Day, Payne, and Dinning, 1950) which was reported in a preliminary experiment to increase tumor incidence when added to low methionine diets. In our laboratory, the inclusion of 100 ug./kilo B_{12} of diet (rice type) was not observed to have any effect on tumor incidence. Pyridoxine deficiency (Miner, *et al.*, 1943) has been

TABLE 2

Activity of Enzymes Concerned in "Labile" Methyl Metabolism in Rat Liver and Liver Tumors

	Choline Oxidase	Methionine Formation (Qm) from Choline	Betaine	DMT
	QO_2			
Liver	6.5	0.09	0.32	69
Liver from Tumor animals	1.9	0.03	0.19	19
Tumor (DMB)	0.1	0	0	0.6

reported to inhibit tumor production, but this effect has been irregularly obtained (Rusch, *et al.*, 1945).

TABLE 3

*Choline Oxidase and Succinoxidase Activity of Rat Kidney Cortex**

	Days on Diet	Choline Oxidase	Succinoxidase
		QO_2	QO_2
Normal diet	.....	3.8	115
Basal diet + 3'MDMB	24–38	3.5	98

*Same rats as shown in Figure III.

Other factors such as the replacement of corn oil by hydrogenated coconut oil (Miller, *et al.*, 1944), detergents such as penetrants 4 and 7 (Miller, Kline, Rusch, 1946) and carcinogenic hydrocarbons such as methylcholanthrene (Richardson and Cunningham, 1951) have also been found to profoundly decrease the carcinogenic activity of the azo dyes. As in the case of the crude protective diets, such as those containing yeast or liver (Kensler, Sugiura, and Rhoads, 1940), both the use of hydrogenated fats and the detergents (Miller, 1947) maintained hepatic riboflavin levels. Those animals developing tumors showed decreased hepatic riboflavin levels. Thus, one might assume (Kensler, Sugiura, and Rhoads, 1940) that hepatic riboflavin levels *per se* would give an indication as to the ultimate protective effect of various dietary supplements. That this is not a valid criterion is shown by the demonstration of the procarcinogenic action of biotin in animals with high hepatic riboflavin levels (Burk, *et al.*, 1943), and by the observation that

TABLE 4

Specie Differences (liver)

	Tumor Production by Azo Dyes	Choline Oxidase	Succinoxidase	DMB Destruction ug/hr.
		QO_2	QO_2	
Rat	high	6.5	79	54
Mouse	low	1.7	84	19
Chicken	none	1.6	50	18
Rabbit	none	0.15	43	19
Guinea pig	none	0	54	30

no significant difference between the protected and non-protected livers was observed when the hydrocarbon carcinogens (Miller, Miller, and Brown, 1952) or alloxan induced diabetes (Salzberg and Griffin, 1952) were employed to inhibit tumor formation.

Choline and cystine (György, Poling, and Goldblatt, 1941) have been shown to afford some protection but not when fed separately. Choline has been reported to increase tumor incidence in one series of experiments (Dyer, 1951). In our laboratory neither choline nor betaine in the presence of folic acid or vitamin B_{12}, or both, afforded protection to rats on a low-riboflavin, rice-type diet. These data are summarized in table 5.

TABLE 5

The Lack of Effect of Folic Acid, Vitamin B_{12}, and Betaine on DMB Carcinogenesis

Diet	Tumor Incidence†
Basal* + 0.06% DMB	10/12
Basal* + 0.06% DMB + 1% Betaine	10/11
Basal* + 0.06% DMB + 100 ug/kg B_{12}	12/14
Basal* + 0.06% DMB + 10 ug/kg PGA	14/16
Basal* + 0.06% DMB + B_{12} + PGA	13/13
Basal* + 0.06% DMB + B_{12} + PGA + Betaine	11/13

*Basal diet—rice, corn oil.
†Animals dying or sacrificed between 120–270 days.

Diets, such as those containing liver (Harris, 1947) or riboflavin and protein (Engel and Copeland, 1949) supplements, which provide good protection against DMB induced liver tumors, are not effective in preventing AAF induced neoplasms. However, riboflavin has been reported to lower the "toxicity" (Wase and Allison, 1950) of AAF in rats, and Bielchowsky (1947) has reported that a diet of bread, skim milk, and yeast delayed the appearance of AAF induced tumors. Engel and Copeland (1952) have observed that rats fed several purified diets show a higher tumor incidence than when several different stock diets were employed. It is noteworthy that in these experiments mammary tumors were the most common and were effected by the diet.

Although it is obvious that our knowledge of nutritional factors that may influence the chemical induction of tumors is far from complete, it is perhaps appropriate to inquire as to the possible mechanism involved in the action of those factors which have been the most extensively studied. Several general possibilities exist. The protection achieved by nutritional means may be related to an alteration in the rate or pathway of metabolism of the carcinogens. In this case, the nutritional effect would be due ultimately to a reduction of the dose of the active carcinogen reaching the effector sites in hepatic cells. However, the effect of diet may also be due to compensation for the intracellular lesions produced by the carcinogen such as the provision of building blocks for resynthesis of damaged components or by the provision of compounds whose synthesis in the cell is prevented by the carcinogens. While it is not possible at this time to explain the effects of diet on the basis of any of the above suggestions, a review of the evidence so far accumulated may be helpful in clarifying the problem.

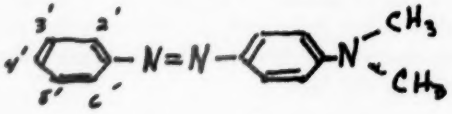
TABLE 6

Enzymatic Activity of Rat and Mouse Liver Tumors as Compared to Normal Liver of the Same Specie

	Rat Tumors			Mouse Tumors		
	Azo	3924	3924A	112B	98/15	10
	%	%	%	%	%	%
Cholin. Ox.	1.7	1.7	0	40	15	55
Succinox.	19	23	13	15	14	57
TB-ase	10	2	3	32	136	79
ChE	174	..	135	43	10	9
DMT	1	..	0.5	20	1	13
DMB	1	..	21	113	26	110

Normal liver = 100.

With respect to the first possibility to be considered, it is now known that the activity of at least some of the enzymes concerned in the metabolism of the azo dyes can be altered by nutritional means. *In vivo*, *DMB* was shown to be demethylated,



reductively cleaved at the azo linkage, and oxidized in the 4′ position by Stevenson, Dobriner, and Rhoads (1942). That at nutritional means. *In vivo*, DMB was shown to be demethylated, least some of the demethylation and the oxidation at the 4′ position occurs prior to the reductive cleavage, who shown by the Wisconsin group (Miller, Miller, and Baumann, 1945; Miller and Miller, 1948). The N-methyl compound is also methylated *in vivo* to form *DMB*. The use (Boissonnas, Turner, and du Vigneaud, 1949) of C_{14} labelled DMB has shown that the bulk of the N-CH_3 is rapidly excreted as CO_2. However, a small percentage is converted into "labile" methyl groups, presumably by resynthesis from formaldehyde or formate (Miller, *et al.*, 1951). That some cleavage occurs prior to complete demethylation was shown by the identification of N-methyl-p-phenylenediamine in the urine. *In vivo* the diets which have been studied do not influence the amount of free dye found in the liver or other tissues but have been shown to effect the amount of bound dye in the liver (Miller and Miller, 1948). It has been suggested that the bound dye is coupled with tissue proteins through its N-methyl group.

In vitro studies disclosed that liver slices, and homogenates, from rats maintained on high-tumor incidence diets of the brown rice type, have a markedly reduced ability to cleave the azo linkage, form less of the 4′ OH derivative, and form proportionately more of the monomethyl derivative (Kensler, 1948; Kensler and Chu, 1950). Supplementation of the rice diet (Kensler, 1948) with liver, yeast, or riboflavin plus casein, which protect against tumor formation, prevents the decrease in rate of cleavage of the azo linkage. An extension of these experiments to rats maintained on a casein-dextrose base diet has shown that while the omission of riboflavin from the diet decreased DMB destruction by liver slices, the omission of thiamin or choline, or the addition of biotin were without effect (Kensler, 1949). A plot of the data obtained on these animals is shown in figure I. It can be seen that those livers containing 15 ug./gm. or less riboflavin destroyed only one-third as much dye as those containing 25 ug./gm. or more. Intermediate riboflavin levels were associated with intermediate dye destroying activity. A more direct demonstration of the involvement of riboflavin as

flavinadeninedinucleotide-FAD) was the finding that FAD must be added to specially dialyzed liver homogenates to get dye destruction (Mueller and Miller, 1950). There is no doubt that a flavoprotein is involved in the metabolism of the azo dyes. It should also be pointed out that the low-protein (four per cent) intake (Kensler, 1949) has also been found to descrease dye destruction but as the liver riboflavin levels are low in these animals, this decrease is presumably due to lowered flavoprotein content. Ekman and Strömbeck (1947) have reported that the metabolism of the azo carcinogen orthoaminoazotoluene is altered by diet. High riboflavin-protein supplements decreased the amount of metatoluidine and increased the amount of the varboxy derivative excreted. They reported that this was associated with alteration in ascorbic acid levels as well as riboflavin levels. An unidentified factor (Miller,

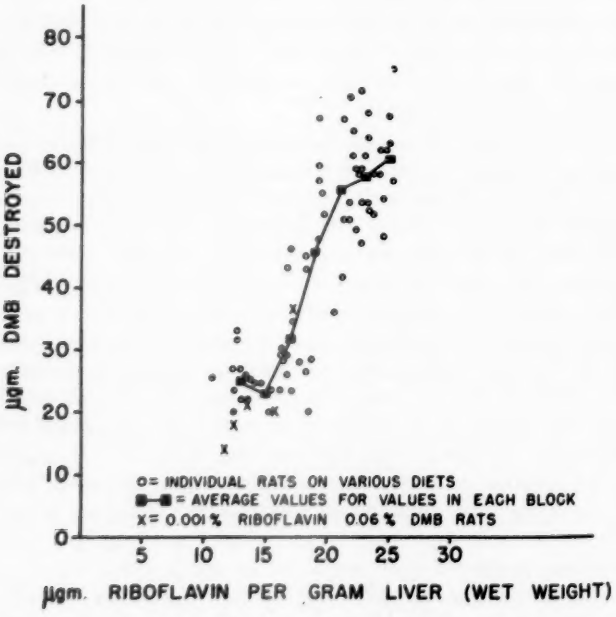


FIG. I. The relationship between the riboflavin content and destruction of DMB by liver slices from rats fed casein-dextrose base diets for 14–20 days.

et al., 1951) in several crude materials such as liver, fishmeal, and reticulogen has been observed to facilitate the oxidative N-demethylation of 3′ methyl-p-monomethylaminoazobenzene.

AAF metabolism has also been reported to be altered by dietary levels of riboflavin supplements (Allison, Wase, and Migliarese, 1950). Increased riboflavin intake is associated with a higher excretion of conjugated AAF and nonvolatile phenols in both dogs and rats. However, tumor incidence has not been found to be influenced by variation in riboflavin intake (Engel and Copeland, 1945). Pantothenic acid levels in the diet also affects the level of conjugated AAF excreted in the urine (Allison, Wase, and Migliarese, 1952).

Thus, while there is evidence that the metabolism of both DMB and AAF can be altered by diet, it is not possible at this time to ascribe with certainty the anti-carcinogenic effect of even high-riboflavin intake to this mechanism. There is, in fact, some evidence which suggests that the preventive effect of dietary supplements, such as yeast or dried milk, for example, is independent of alterations in the metabolism of the carcinogen. Sugiura's early data (Kensler, 1947) and its recent confirmation (Sugiura, 1951) indicate that there is a period in the induction of tumors when the process is reversible and that tumors are formed or not depending on the diet fed after the administration of the carcinogen is stopped. As the rats, previous to this point, had been metabolizing approximately 6000 ug. of dye per day, and as only a few micrograms are present in the liver and other tissues at this time, any further effect of diet on the metabolism of the carcinogen would appear to be excluded. There is no information available on the level of bound dye in the liver at this point or on the effect of these diets on its rate of disappearance.

A number of years ago it was suggested (Kensler, 1947) that the azo carcinogens might produce liver tumors as an end result of damage to some essential enzymatic system by dye metabolites formed in the liver. Although a variety of hepatic cell enzymes are decreased in activity during the administration of the azo dyes, there is no definite evidence that these changes bear any causal relationship to the formation of neoplastic cells. However, the demonstration by the Alabama group (Copeland and Salmon, 1946; Schaefer, Copeland, and Salmon, 1949) that

liver tumors and tumors of other tissues can be produced by maintenance of rats or chickens in a state of chronic choline deficiency, suggested the desirability of securing data on enzymes involved in choline and "labile" methyl group metabolism (Fig. II). If choline or "labile" methyl group metabolism were found

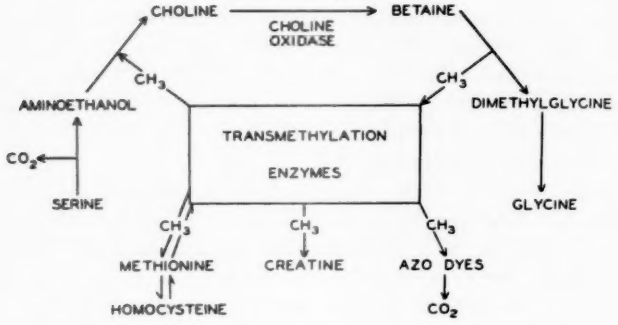


FIGURE II.

to be selectively interfered with by the carcinogenic azo dyes, it would suggest that the induction of liver tumors by the dyes might in fact be due to the production of a local "induced" deficiency (Kensler and Langemann, 1951) in hepatic cells similar to that produced when rats are maintained on the Alabama group diets. One observation (Schaefer, *et al.*, 1950) that supports the idea that induction of tumors by azo dyes or choline deficiency may have some pathway in common is the finding that high-riboflavin supplements protect against tumor formation in both cases. In addition, the azo dyes may be acting to further deplete "labile" methyl group donors by acting as methyl group acceptors. This would in effect amount to a "labile" methyl group wasting mechanism as the methyl groups picked up by the monomethyl azo dye are oxidatively removed and hence lost to the animal for direct use in transmethylation actions.

In the rat, Dubnof (1949) and Muntz (1950) have provided evidence that choline must be oxidized to betaine by the choline oxidase system before its one transferable methyl group is available for transmethylation reactions. Hence, any inhibition of the

activity of this system could be expected to duplicate the effects of low-choline intake, at least insofar as the supply of labile methyl groups in the liver is concerned. Choline oxidase activity is also decreased in livers of rats on a riboflavin (Viollier, 1948) or protein (Kensler and Rudden) deficient diet, and it has been reported to be elevated by high-riboflavin intake (Kelly, 1952). This supplies another possible site of action to explain the riboflavin protection against tumor formation.

It is therefore of interest that choline oxidase activity has been found to be extremely low or absent in rat liver tumors (Lan, 1944; Viollier, 1950; and Woodward, 1951). We have found (Kensler and Langemann, 1951) little or no choline oxidase activity in liver tumors produced by 3′ M-DMB. The data are shown in table 2. A study also has been made of the effects of 3′ M-DMB feeding on choline oxidase activity as a function of the duration of feeding. These data are shown in figure III. Succinoxidase activity was also measured as it was shown to be reduced by 3′ M-DMB administration (Potter, *et al.*, 1950) and as both enzyme systems (Hogeboom, Schneider, and Pallade, 1948; Kensler and Langemann, 1951 are to a large

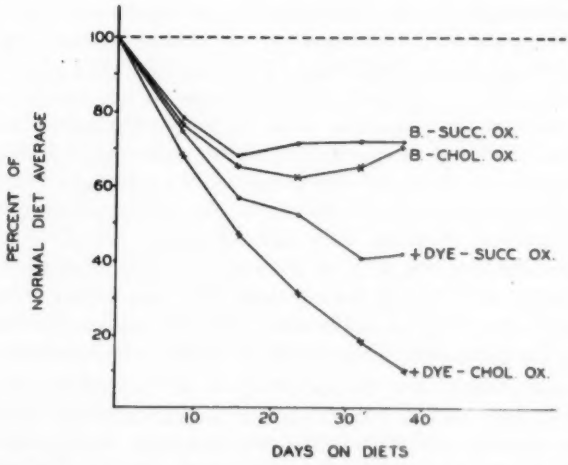


FIG. III. Choline oxidase and succinoxidase activity of the livers of rats fed 3′ methyl-p-dimethylaminoazobenzene.

extent associated with the nitrochondrial fraction of liver cells. This fraction is grossly depleted in rat liver by the carcinogenic azo dyes (Price, *et al.*, 1950). The rats were fed a high-tumor incidence basal diet of rice and 18 per cent casein, but no riboflavin supplement was given. The activity of both succinoxidase and choline oxidase decreased in the absence of the dye but leveled off after three weeks. However, when the dye was included, both enzymes decreased to a greater extent and choline oxidase more so than succinoxidase. In the kidneys of the same animals, neither succinoxidase nor choline oxidase activity were decreased by the diet alone or with dye included as is shown in table 3. It would thus appear that the effect of the carcinogen on these enzymes *in vivo* is restricted to the tissue where the tumors ultimately appear.

One of the striking things about both azo dye carcinogenicity and hepatic choline oxidase activity is the marked specie differences encountered. In table 4 the data obtained on several species are summarized. It can be seen that there is no great difference among the various species examined with respect to succinoxidase activity in DMB (reductive cleavage) by liver slices, but that choline oxidase activity varies markedly. Both the guinea pig and the rabbit, which are highly resistant to azo dye carcinogenesis, have little or no choline oxidase activity; and the rat, which is the most susceptible, has the highest level of choline oxidase activity. However, the chicken (adult, white leghorn) which is resistant, has appreciable choline oxidase activity. Although the guinea pig and the rabbit have little or no choline oxidase activity, the transferable methyl group of choline is still apparently utilized for transmethylation reaction in these species (Dubnoff, 1949; Schenk, *et al.*, 1943). Thus, one might postulate that only in those species which are dependent on choline oxidase will the azo dyes exert their effect. The dog, which is not included in the table, also has appreciable choline oxidase activity and is susceptible to DMB carcinogenesis.

If one assumes that the inhibition of choline oxidase *per se*, as distinct from an interruption of the "labile" methyl "cycle," is an important step in azo dye carcinogenesis, then, as betaine formation would be blocked, one might expect that the addition of this compound to the diet should protect against the action

of the azo dyes. As is indicated in table 5, under the conditions studied, this has not been found to be the case. Thus, one might say that interference with "labile" methyl transfer is not involved in the action of the azo dyes unless enzymes other than choline oxidase, and involved in methyl group metabolism, are also inhibited during azo dye feeding. The ability of liver to transfer a methyl group from choline, betaine, and its sulphur analog, dimethylthetin, to homocysteine to form methionine has therefore been measured. It has been found (Table 2) that these processes also are not carried out to any detectable extent by the liver tumors produced by dye feeding and that they are depressed in the pre-cancerous liver tissue. Thus, the supply of labile methyl groups provided by feeding betaine also would not be available for use in the dye-fed animals. However, these results do indicate that the simple assumption that choline oxidase is the critical enzyme involved in azo dye carcinogenesis is not tenable.

Further evidence against any direct connection between low choline oxidase activity and liver tumors is provided by our study (Kensler, Rudden, and Langemann, 1952) of the enzymatic activity of three transplantable mouse hepatomas, 112B, 98/15, and azo dye induced hepatoma 10. Several enzyme systems were measured in these tumors and in two transplantable rat liver tumors (3924, 3924A) originally induced by AAF. The data are summarized in table 6. It can be seen that whereas all three rat tumors had little or no choline oxidase activity, that the mouse hepatomas contained from 15 to 55 per cent of the activity of normal mouse liver. The activities of succinoxidase, tributyrinase, cholinesterase (ChE), and DMB destruction varied markedly among these tumors. However, dimethylthetin (DMT) tended to be low in the liver tumors of both species.

One interesting aspect of the low dimethylthetin transmethylase activities in all of the liver tumors examined is the observation that livers of newborn rats possess the same order of activity as adult rat livers. This is in marked contrast to choline oxidase activity which is low in both the livers of the newborn rats and rat liver tumors. The data are summarized in figure IV. It can be seen that succinoxidase activity (SO) starts low and increases rapidly, and choline oxidase activity (ChO) starts low and in-

creases slowly (Kensler, *et al.*, 1952). However, both dimethylthetin transmethylase activity (DMT) and betaine transmethylase activity (Bet) are high at birth. Thus, the data indicate a marked difference in transmethylase activity between young liver tissue and liver tumors and suggest that an action on "labile" methyl group metabolism may be implicated in the carcinogenic activity of the azo dyes.

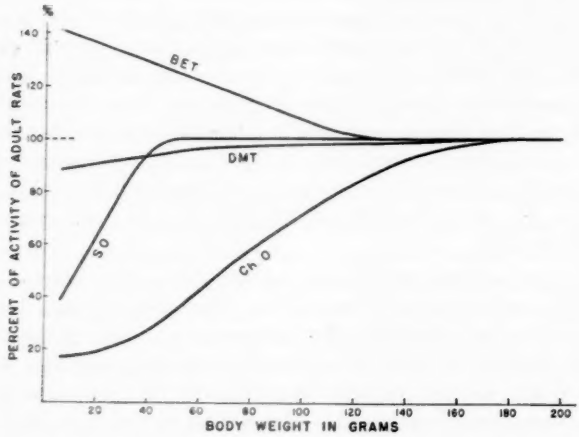


FIG. IV. Activity of rat liver enzymes as a function of body weight. BET —betaine transmethylase, DMT—dimethylthetin transmethylase, SO— succinoxidase, ChO—choline oxidase.

In summary then, although much essential information remains to be acquired, it appears likely that nutritional effects on the chemical induction of tumors are not due exclusively to alterations in the metabolism of the carcinogen. While no conclusive evidence has been obtained linking azo dye carcinogenesis and the production of tumors by chronic choline deficiency, further studies in this area should be rewarding.

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TIME AS A FACTOR IN THE CALORIC UNDERFEEDING EFFECT UPON CANCER*

JOSEPH T. KING

It is now generally accepted that the amount and kind of food eaten by cancer-susceptible mice may to a large degree determine the incidence of mammary tumors and the age at which these appear.

Woolley, Fekete, and Little (1941) reported that certain strains of mice develop a state of continuous sub-estrus after ovariectomy. King, Casas, and Visscher (1949) reported that the characteristic cellular changes develop in the adrenals following ovariectomy whether on full feeding or one-third caloric restriction. However, the restricted mice failed to show the evidences of estrogen production noted in the full-fed groups.

In the present investigation, C_3H mice, ovariectomized at 21–23 days of age, were full-fed for varying periods and then placed on caloric restriction. Observations were then made over the life span on the development of mammary tumors. In this way we hoped to learn whether a critical amount of full feeding was required to produce a state subsequently leading to development of mammary carcinoma, *i.e.*, a state not reversible by caloric restriction.

Similar mice were started on restriction and were later placed on full-feeding at various intervals to determine whether limited periods of restriction early in life produced any lasting inhibition of the development of mammary tumors usually seen on full-feeding. A group full-fed over the whole life span and a group similarly restricted were observed as controls.

The mice were kept in an air conditioned room at 78° ± 4° F. Tap water was available at all times. They were individually housed in sheet metal screen cages. Those being fed ad libitum were given a fresh food cup weekly. Those on restriction were fed the required amount daily. The diet used was that described by Visscher, Ball, Barnes, and Sivertsen (1942). The restricted

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mice were fed approximately the same amount of protein, vitamins, and minerals eaten by the controls, but a reduced amount of fat and carbohydrate. The caloric restriction was approximately one-third.

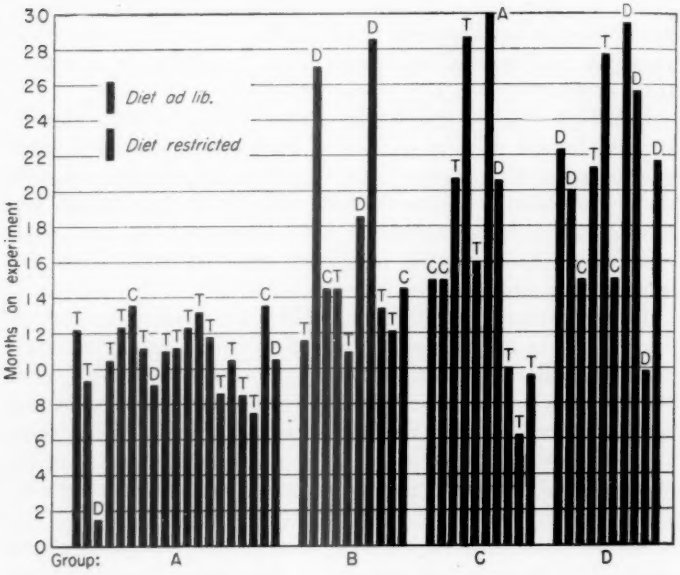

FIG. I. Life span and mammary tumor incidence in ovariectomized C$_3$H mice on full-feeding (Group A) and full-feeding for various periods before reversal to one-third restriction as indicated (Groups B, C, and D). T indicates death due to mammary tumor; D, death due to other cause; C, removed for other study, tumor-free; A, alive at 30 mos., died tumor-free.

Results

In figure I are shown the results of the experiment in which the mice were started on full-feeding. Group A was kept on full-feeding for the life span; groups B, C, and D were full-fed for 6, 3, and 2 months respectively before being reversed to restriction. The life span is shown for each mouse. Whether the mouse developed a tumor, died of other cause, or was removed for chemical analysis is indicated.

It will be seen that the tumor incidence is high for all groups full-fed for 3 months or more. In group D, full-fed for only 2 months, the incidence is low and the average tumor age much higher than for groups A, B, and C. In table 1 tumor incidence and average tumor age are shown for the four groups started on full-feeding. (The two mice removed for carcass analysis have not been counted in the total; they were tumor-free when killed.)

TABLE 1

Type of Feeding	Tumors	Av. Tumor Age
Full, life span	14/17	10.7 mos.
Full, 6 mos., then restricted	5/8	12.5 mos.
Full, 3 mos., then restricted	6/8	15.2 mos.
Full, 2 mos., then restricted	2/8	24.5 mos.

The results of the experiment in which mice were started on restriction are shown in figure II. Here the groups are arranged according to increasing periods of restriction, *i.e.*, for 2, 3, 6, and 8 months, and life span. Data is shown in figure I.

Tumor incidence and average tumor age for the five groups are shown in table 2.

TABLE 2

Type of Feeding	Tumors	Av. Tumor Age
Restricted 8 mos., then full	6/8	14.2
Restricted 6 mos., then full	4/7	14.8
Restricted 3 mos., then full	4/8	11.7
Restricted 2 mos., then full	6/7	11.7
Restricted life span	1/7	10.4

It is evident that the castrate retains the capacity for high tumor incidence on reversal to full-feeding even after eight months of restriction under these experimental conditions. It is believed the single mouse developing a tumor in the life restricted group is a random exception since we have observed tumors only rarely under these conditions in other experiments.

Discussion

Recent experience with statistical analysis of groups of various size leads us to believe that we would have obtained more

uniform results with groups of 24 mice. Even granting some irregularity due to the use of small numbers, it seems clear from the data shown in table 1 that a relatively short period of ad libitum feeding induces a state which cannot be controlled by subsequent restriction (known to be adequate to suppress development of tumors in all but rare instances when such restriction is started at weaning).

Data in figure II show that all groups reversed to full-feeding developed high cancer incidence. The periods of restriction studied provide no protection against the development of mammary carcinoma on subsequent refeeding (unless one interprets the apparent increase in tumor age as limited protection).

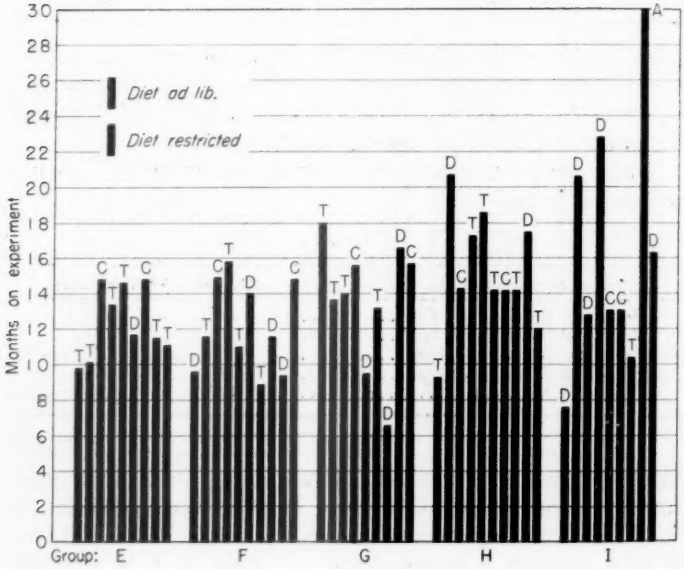

FIG. II. Symbols as in figure I. Groups E, F, G, and H were reversed from restriction to full-feeding as indicated; Group I restricted over life span.

It is obvious that additional groups require study. Especially we need to determine the results of shorter periods of prelim-

inary full-feeding and longer periods of preliminary restriction. The work is in progress using larger groups.

Conclusions

Periods of preliminary full-feeding as short as 3 months followed by restriction induce a high cancer incidence in castrate C_3H mice.

Periods of preliminary restriction as long as 8 months fail to protect such mice against high cancer incidence when the mice are subsequently fed ad libitum.

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NUTRITIONAL AND HORMONAL INTERRELATIONSHIPS IN THE DEVELOPMENT OF EXPERIMENTAL CANCER

HAROLD P. MORRIS*

Comparatively little is known of the effects of prolonged specific nutritional deficiencies on hormonal secretions. Would prolonged changes in the concentration of a hormone increase or decrease the utilization of particular dietary factors? Would partial deficiency of one or more dietary factors alter hormonal secretions? The posing of such problems and the search for their solution open up a comparatively unexplored frontier between nutrition and endocrinology. The long-continued complete or partial dysfunction of an endocrine gland caused by a dietary inadequacy may have far-reaching consequences in the study of many biological and medical problems, including the origin of some kinds of cancer.

An understanding of the genesis of cancer should give consideration to environmental factors that influence either directly or indirectly the cytoplasm, the genes, and enzyme systems. Although nutrition alone may not explain the entire mechanism, it is an important tool which has been and will continue to be utilized in attempts to obtain more knowledge of factors influencing the development of cancer.

I expect to discuss the way in which deficiencies of some nutritional constituents may alter the hormonal balance in the intact animal primarily from the standpoint of metabolism, *i.e.*, the sum of the processes concerned in the building up and breaking down of protoplasm incidental to life processes. Since nutritional deficiencies alter metabolic processes going on within the body, the inclusion of antimetabolites which interfere with specific metabolic processes logically comes within the field of nutrition and provides a new procedure to study nutritional problems. The effects of one specific antimetabolite, thiouracil, which has been used in our laboratory for several years to

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study the relation of hormonal balance on thyroid and spontaneous mammary cancer development in mice, will be presented and its effects contrasted with the effects produced by some nutritional deficiencies.

Thiouracil and similar antithyroid agents produce a condition in the animal which may be described as chemical thyroidectomy (Morris, Dubnik, and Dalton, 1946). Such a state specifically results in an inhibition of the function of thyroxine formation, but in the presence of an intact pituitary, produces a stimulation of the thyroid gland (MacKenzie, C. G., and MacKenzie, J. B., 1943 and 1944; Astwood, *et al.*, 1943). It differs from surgical thyroidectomy by not physically removing the thyroid, thus probably leaving intact other metabolic functions of this gland. The use of these agents, therefore, makes available a technique for evaluating some specific metabolic responses and allows a comparison of these with responses of the animal to various types of nutritional deficiencies.

What evidence now exists to explain the action of these different types of deficiencies upon the development of mammary cancer in mice? Extensive growth of the lobule alveolar system of the mammary glands seems to be an essential preparatory stage to the formation of mammary adenocarcinoma in the mouse. Therefore, any dietary regimen which inhibits such development becomes a valuable experimental procedure for delaying, partially inhibiting, or even preventing mammary cancer in mice.

Chronic underfeeding has been shown by Tannenbaum (1940) to reduce spontaneous mammary tumors in susceptible dba strain mice. Visscher, *et al.* (1942) and White, *et al.* (1944) have observed that a more specific deficiency, chronic caloric restriction, in female strain C3H mice inhibits or prevents the formation of spontaneous mammary tumors. White, *et al.* (1943 and 1944) have also found that the amino acid deficiencies of cystine and lysine inhibited spontaneous mammary tumor genesis in strain C3H mice, and Morris (1947) has reported a reduction in mammary tumor incidence in the same strain after prolonged partial ariboflavinosis. Table 1 summarizes some of these results. It is obvious from the table that a variety of nutritional regimens produce the same end-

TABLE 1

Some Nutritional Inadequacies Effective in Decreasing or Preventing Spontaneous Mammary Cancer in Female Strain C_3H Mice

Lit. Ref.	Type of Regimen		Mammary Gland	Estrus	Tumor Incidence Control	Exp.
					%	%
Visscher (7)	1/3 Restriction Calories	Virgin	.....	.....	67.0	0.0
White (8) (12)	1/2 Do	Do	Infantile	Absent	100.0	12.5
White (8) (12)	1/2 Do	Breeding	Atrophy	.....	100.0	18.2
White (10) (12)	Lysine Def.	Virgin	Infantile +	Occasional	97.6	25.0
White (9)	Cystine Def.	Do	Infantile	Absent?	97.4	0.0
White (11) (12)	Cystine Def. + Stilbesterol	Do	Complex	Continuous	92.0	45.0
Morris (13)	Riboflavin Def.	Do	Infantile	No. Effect?	98.0	66.0

result, *i.e.*, they partially or completely inhibit the development of mammary cancer in mice. The severity of a nutritional restriction and the amount of breeding can be seen to be important factors affecting the incidence of mammary tumors. The most effective regimens were those begun on young virgin mice and were continued until death, although mice that were severely restricted in calories after raising one or two litters also showed a definite decrease in tumor incidence. The effect on estrus and mammary gland development in some experiments was striking (White, 1944). Estrus was absent in the mice severely restricted in calories. There was also a complete or partial inhibition of mammary gland development in deficient animals in which estrus was absent or took place only occasionally (White, 1944). It should be kept in mind that all mice fed the restricted or deficient diets possessed (a) a susceptibility to mammary-tumor formation inherent in the genetic constitution of the animal, (b) the so-called milk influence which is normally transmitted to the offspring through the mother's milk, and (c) the hormonal system, whose influence is known to be essential for mammary cancer formation in this species.

The hormones stimulating estrus and the development of the mammary tissue appear to be important factors influenced by diet. Estrogenic hormone production is one factor affected, because the administration of large amounts of diethylstilbestrol to cystine-deficient mice (table 1) induced continuous estrus and stimulated the development of the mammary glands. Increasing the estrogenic stimulation to levels above normal levels by diethylstilbestrol (White, 1944), however, only partially overcame the effects of the low cystine diet. Huseby and associates (1945) examined histologically ovaries of mice after one-third restriction in caloric intake for 17–18 months, and concluded that partial inanition preserved the ovary in a pseudo-juvenile condition that resembled what Moore and Samuels (1931) and Mulinos and Pomerantz (1940) have described as pseudohypophysectomy. A quite similar condition of the ovaries has recently been observed by Morris, Dunn, and Wagner (1952) in young dba mice deficient in pyridoxine and in paired pyridoxine sufficient mice restricted in food in-

take (Fig. I). The weights of the ovaries of pyridoxine-deficient and diet-restricted animals shown in figure I were much less than weights of ovaries in mice receiving *ad libitum* a diet adequate in pyridoxine. The absence of luteal tissue was noteworthy. The ovaries in pyridoxine-deficient and paired diet-restricted animals responded to injections of pregnant mares serum gonadotrophic hormone by a marked increase in weight and showed definite evidence of luteal tissue formation. This we interpret as indicating an inability of the anterior pituitary of pyridoxine-deficient and diet-restricted mice either to produce or to release adequate amounts of gonadotrophic hormone. The target organ, the ovary, is still able to respond to

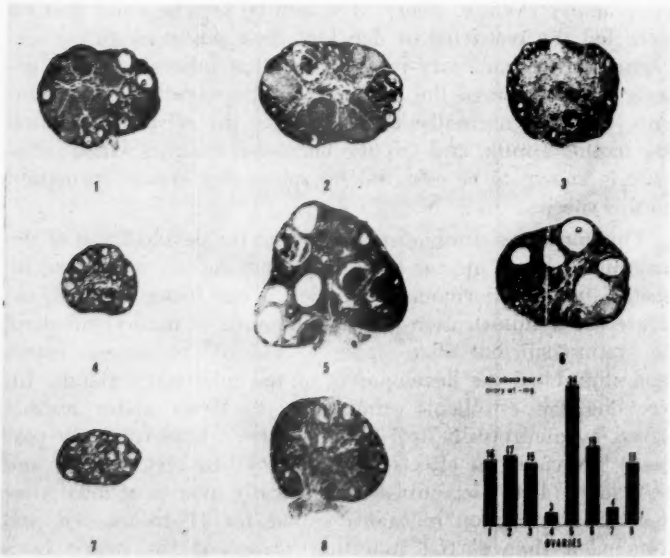


FIG. I. Cross section of ovaries of dba mice pyridoxine deficient. 1. Control + pyridoxine, 2. Control + pyridoxine + gonadotrophin for three weeks, 3. Control + pyridoxine + gonadotrophin for eight weeks, 4. Pyridoxine deficient, 5. Pyridoxine deficient + gonadotrophin three weeks, 6. Pyridoxine deficient + gonadotrophin eight weeks, 7. Pyridoxine supplemented but restricted in food to amount consumed by animals in group four, 8. Pyridoxine supplemented but restricted in food to amount consumed by animals in group five. Bargraph—average weights of ovaries of mice in each group.

exogenously supplied gonadotrophin. Our experiments were of too short duration, however, to give any evidence on the influence of gonadotrophic hormone injections on the incidence of spontaneous mammary tumors.

Dietary or nutritional inadequacies as described thus far indicate an insufficiency of the secretions of the ovary and of the anterior pituitary gland. Let us now examine the effects in mice following prolonged administration of the goitrogenic drug, thiouracil, and compare the effects of specificially inhibiting the secretion of thyroxine by the thyroid gland through the use of this antimetabolite to the conditions produced in mice by caloric restriction or certain dietary or nutritional inadequacies. Table 2 compares the organ weights of control

TABLE 2

Organ Weights of Control and Experimental Animals. Control Diet 77—Stock Diet Composed of Natural Foodstuffs. Experimental Diet 145 Above Stock Diet Containing 0.37% Thiouracil the First Six Months Then the Thiouracil was Increased to 0.5%

Organ	Number of animals	Average weight	Range
		mg.	*mg.*
CONTROL DIET 77			
Thyroid	7	4.5	2.6– 7.0
Thyroid and trachea	9	14.9	11.2– 22.5
Ovary	10	7.4	3.2– 12.5
Ovaries and uterus	26	116.5	30.2–242.0
Adrenals	27	7.2	3.9– 12.2
EXPERIMENTAL DIET 145			
Thyroid	53	59.3	12.3–141.5
Ovary	9	4.7	2.0– 7.3
Ovaries and uterus	38	36.3	13.3–110.0
Adrenals	38	4.1	2.1– 6.0

mice fed a stock diet to the organ weights of experimental mice fed the same diet to which had been added three-eighths to one-half per cent of thiouracil (Dalton, Morris, and Dubnik, 1948). The average weight in milligrams of thyroid glands of the control animals was 4.5, and that for the treated animals, 59.3. The ovaries of control animals averaged 7.4 milligrams in weight while the ovaries of treated mice were only about

half as heavy, weighing 4.7 milligrams. The combined ovaries and uterus weight for control mice was 116 milligrams while for the thiouracil-treated animals it was only 36 milligrams.

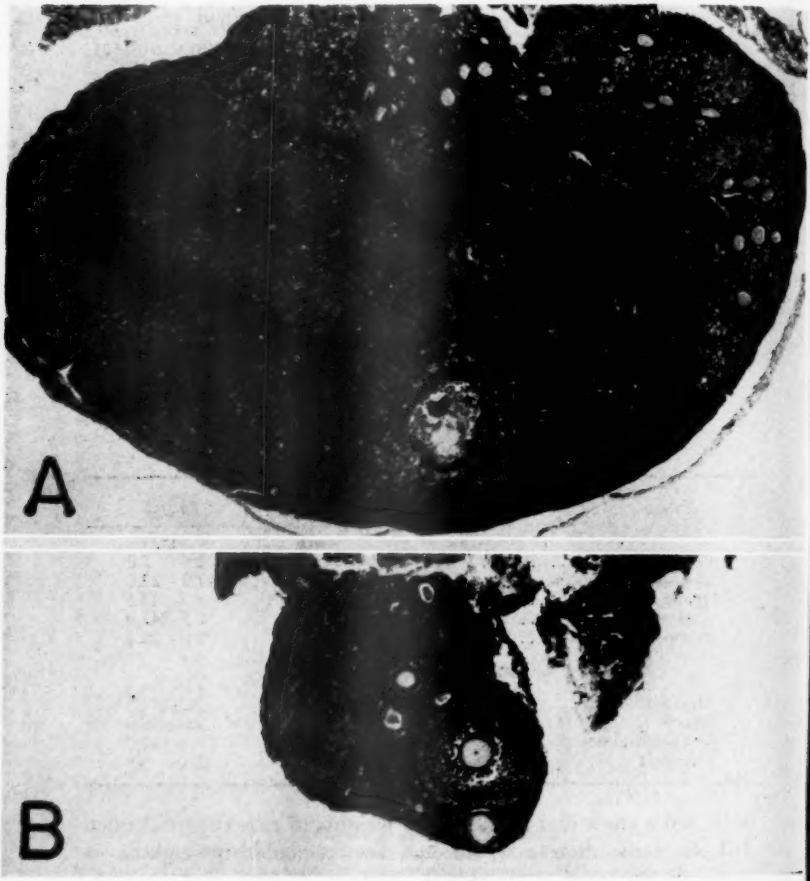


FIG. II. A. Section of ovary from mouse, 352 days of age, on control diet showing immature ova, follicles, corpora lutea, and interstitial cells. X 55. B. Ovary from a mouse treated with thiouracil for 464 days, showing immature ova, and developing follicles; corpora lutea and interstitial cells absent. X 65. Both A and B fixed in Zenker-formol and stained with hematoxylin and eosin.

The adrenals in the treated animals were also about half as large as the adrenals in control animals.

A normal ovary from a control mouse is illustrated in figure II A, showing well formed corpora lutea, and II B, an ovary of a thiouracil treated animal with immature ova and developing follicles but no corpora lutea or interstitial cells (Dalton, Morris, and Dubnik, 1948).

The number of estrus phases per month for control and thiouracil treated mice is presented in figure III (Dubnik, Mor-

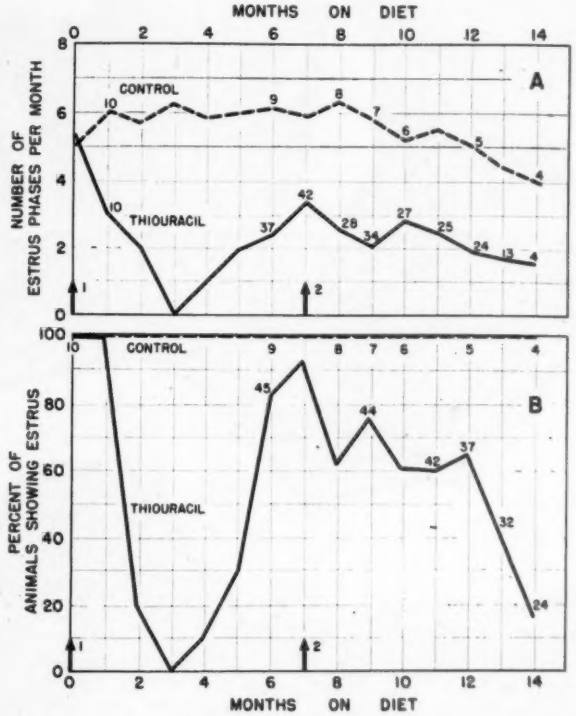


FIG. III. A. Frequency. B. Incidence of vaginal cornification in control and thiouracil treated mice. Arrow 1:0.375 per cent thiouracil added to stock diet. Arrow 2: amount of thirouracil increased to 0.5 per cent. The number above or below each point represents number of animals used to obtain value.

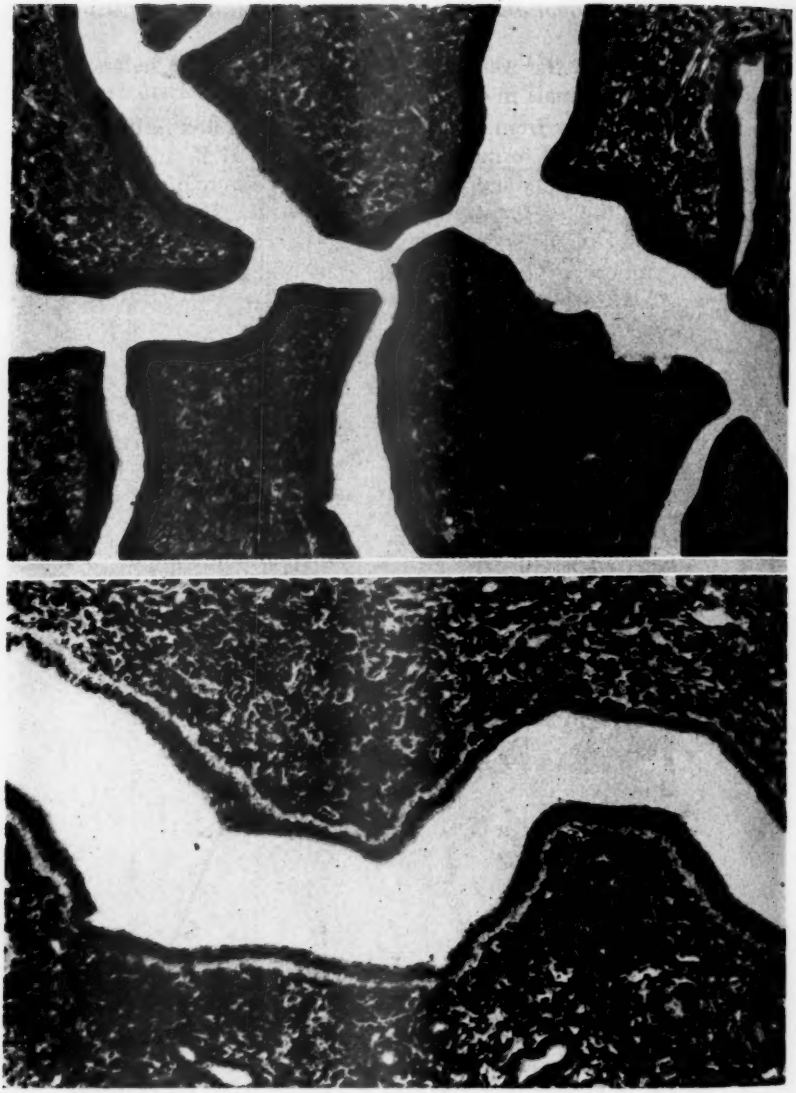


FIG. IV. A. Endometrium of uterus of control mouse 412 days of age. B. Endometrium of mouse treated with thiouracil for 440 days, showing a thin inactive epithelium and relatively few, shallow glands. Both A and B fixed in Zenker-formol, stained with hematoxylin and eosin. X 200.

ris, and Dalton, 1950) at the top. Throughout the first nine months of the experiment, the control animals were in estrus approximately six times per month; thereafter, estrus frequency dropped somewhat. Estrus frequency in the experimental group dropped immediately after thiouracil ingestion, reaching zero by the third month. There was, however, a gradual recovery of estrus in these animals as noted by an increase in frequency during the next three months to about half that of the control animals. The percentage of animals showing estrus in the control group to that of the thiouracil-treated group is shown in the lower part of the figure (B). The control group maintained 100 per cent estrus throughout the period of administration of thiouracil.

The endometrium of the uterus of a normal control mouse 4.2 days of age is shown in figure IV A, and figure IV B, the endometrium of a mouse treated for 440 days with thiouracil. The endometrium of the treated animals showed fewer and less complex glands than did the controls (Dalton, Morris, and Dubnik, 1948). The percentage of control and experimental

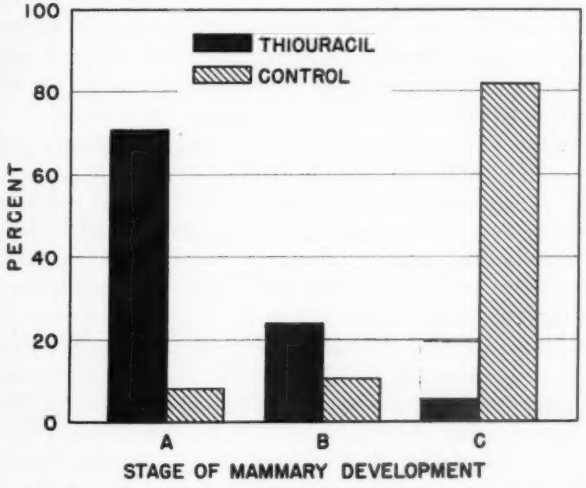


FIG. V. Percentage of control and thiouracil treated animals possessing different stages of mammary development. A—simple, B—intermediate, C—complex.

mice that possessed (a) simple, (b) intermediate or (c) complex development of the mammary glands is illustrated in figure V (Dubnik, Morris, and Dalton, 1950). It is readily apparent that the majority 82 per cent, of control animals achieved complex development while the majority of the treated animals, 71 per cent, never progressed beyond the most simple kind of mammary architecture. Intermediate type of development was achieved by 11 per cent of the control and 24 per cent of the treated animals. As shown in table 3, 92 per cent

TABLE 3

Effect of Thiouracil on Age of Appearance and Incidence of Mammary Tumors

Diet	No. of Mice	Tumor Incidence	Age when Tumors appeared	Average age of Tumor appearance
		Percent	Days	Days
77 (Stock)	53	92	187–529	330
145 (Thiouracil)	52	19	189–609	391

of the control animals developed mammary tumors while 19 per cent of the animals treated with thiouracil developed tumors (Dubnik, Morris, and Dalton, 1950). Thiouracil-treated strain C mice in a subsequent experiment developed no mammary tumors (Morris, Green, and Dalton, 1951).

A scatter diagram of the thyroid weights in C3H mice plotted against the length of time the thiouracil was administered is shown in figure VI (Dalton, Morris, and Dubnik, 1948). This diagram shows that in general thyroid gland weight was proportional to the length of treatment. However, there was considerable amount of individual variation, particularly in animals treated for more than 300 days.

The colloid in the thyroid gland during the early period of thiouracil feeding (Dalton, Morris, and Dubnik, 1948) lost its acidophylic staining reaction and decreased in amount, first in the center of the gland and later at the periphery. The cells increased in size and acquired a definitely columnar form. The nuclei became larger and relatively vesicular and, hence, pale blue in hematoxylin and eosin preparations. In spite of extreme hypertrophy of the thyroid gland involving both cellular

hypertrophy and hyperplasia, resulting in some instances in increases to as much as 30 times the normal weight, no histological evidence of neoplasia was found. With continued thiouracil ingestion, the thyroid retained this hyperplastic state for approximately twelve months (Dalton, Morris, and Dubnik, 1948), but at about this time a few follicles were found to contain a moderate amount of acidophilic colloid. Several mice exhibited numerous superficial pearly gray nodules in their lungs after twelve to fifteen months of thiouracil ingestion (Dalton, Morris, and Dubnik, 1948 and 1946). All such nodules were identified as thyroid tissue remarkably similar in histologic appearance to that of the host thyroid, figure VII (Dalton, Morris, and Dubnik, 1948).

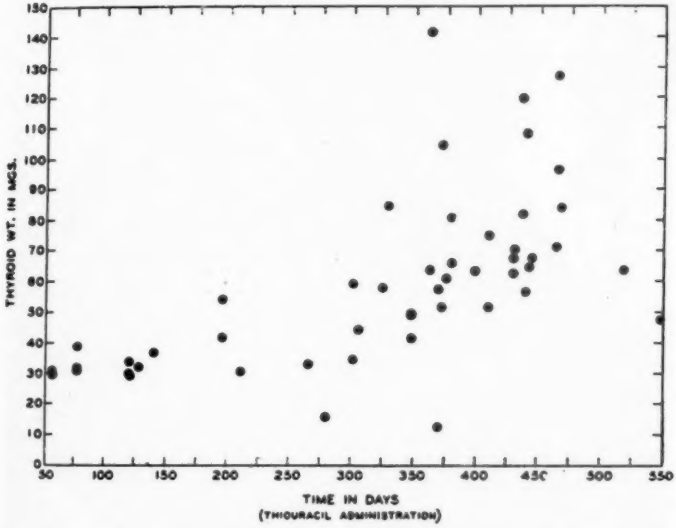


FIG. VI. Scatter diagram showing variation in thyroid weight in relation to length of treatment with thiouracil.

A schematic diagram to explain a possible mechanism by which thiouracil inhibits mammary tumor formation is presented in figure VIII (Dubnik, Morris, and Dalton, 1950). The pituitary gland in its attempt to secrete more and more thyro-

FIG. VII. Pulmonary metastasis of thyroid tissue in a mouse given thiouracil for 464 days. Chromophilic nuclei in cells lining colloid containing follicle. X 355.

trophin during thiouracil ingestion may not maintain its secretion or release of the other tropic hormones at a level sufficient to stimulate adequate functioning of other indocrine glands. Although the thyroid glands increase in size, the thyroxine insufficiency continues. The pituitary maintains the production of large amounts of thyrotrophin, TSH, but only at the expense of other trophic hormones—lower quantities of gonadotrophin and possible mammaotrophin are present in the circulation. This lack of gonadotrophic stimulation depresses ovarian function resulting in a reduced output of estrogen. Thus, the hormone specifically concerned with mammary growth, estrogen, and also mammaotrophin may be produced in insufficient amounts; and the general metabolic hormone, thyroxine, is entirely absent. Consequently, the mammae fail to develop fully, hyperplastic nodule formation is reduced, and finally there is a depression of mammary tumor genesis.

Partial chronic inanition in mice has been shown by Barton and Rusch (1952) to produce at least a 50 per cent increase in the oxidation of glycine to respiratory CO_2, suggesting that there is an increased conversion of the dietary protein to energy. Such diversion of protein, if extensive, during chronic inanition could cause a hormonal imbalance in at least two ways, directly by deamination creating an insufficiency of amino acids to allow synthesis of tissue proteins for growth including mammary gland growth or indirectly through failure to secrete or release protein-containing pituitary hormones. Even though there were found decreases in size of some organs influenced by trophic hormones, total failure of pituitary hormone secretion apparently does not occur in chronic thiouracil-treated mice because the thyroid glands of treated mice were greatly enlarged indicating increased production of TSH. The MacKenzies (1943 and 1944) and others have shown thyroid enlargement with goitrogenic drugs occurs only in the presence of the hypophysis. A deficiency of gonadotrophic secretion on the other hand was evident because mammary development did take place in a few animals to whom we administered thiouracil plus gonadotrophin (Dubnik, Morris, and Dalton, 1950). Normal estrus also appeared in the gonadotrophin-treated mice indicating the target organ, the ovary, was still

able to function when adequate pituitary stimulation was present.

The inhibition of mammary tumor genesis in several types of nutritional deficiencies and in thiouracil-treated mice appears to be produced through the effects on hormonal secretions. While it is believed that hypophyseal insufficiency is the controlling factor in both nutritional and thiouracil-produced hormonal imbalances, the mechanism may not be identical. It was pointed out that blocking the secretion of the thyroid gland by the antimetabolite, thiouracil, produces striking effects on several organs and tissues and culminates in inhibiting or preventing the development of spontaneous mammary cancer in mice. The hormonal imbalance occurs differently, however, during thiouracil administration than in the nutritional deficiencies thus far studied because the thyroid tissue during thiouracil ingestion is stimulated by the excessive TSH secretion, a condition not observed to occur in nutritional deficiencies.

Hypertrophy and hyperplasia of the thyroid tissue occurs, and metastatic lesions of thyroid tissue appear in the lungs with prolonged thiouracil ingestion, due, we believe, to excessive TSH secretion. Histologically, these thyroid metastases (Dalton, Morris, and Dubnik, 1948) resembled the host thyroid at autopsy (Fig. VII). We have, however, successfully transplanted subcutaneously lung metastatic nodules of thyroid tissue in their entirety from two different mice. These transplants were made into young hosts that were also ingesting thiouracil. The metastatic transplants were at first dependent growths because they grew only in mice ingesting the goitrogen. They were transplanted to new hosts at intervals of three or more months depending on the size the transplants attained. Complete growth autonomy, *i.e.*, ability to grow without TSH stimulations, now has been obtained in both these lung nodule lines, one in the fourth, the other in the seventh transplant generation. Other lines of transplantable thyroid tumors have been established (Morris and Green, 1951) by transplanting the hyperplastic thyroid glands of mice after ingesting thiouracil for sixteen to eighteen months. Growth autonomy has also occurred in the third and ninth transplant generations in two lines, but a third line is still dependent on

thiouracil ingestion even after eleven generations of transplantation (Morris and Green, 1951; and Morris, 1952).

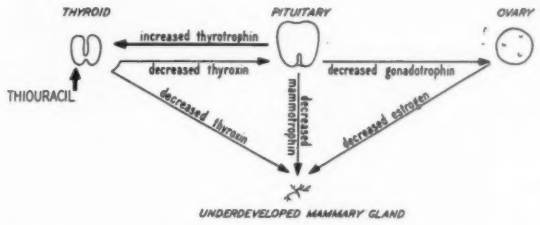


FIG. VIII. Schematic diagram designed to help interpret the syndrome produced by prolonged feeding of thiouracil to C_3H female mice.

The next few illustrations show some of the results of this phase of our studies on hormonal imbalance produced by prolonged ingestion of thiouracil (Morris, Dalton, and Green, 1951).

Figure IX shows the thyroid of a host carrying a ninth generation transplant. This mouse had not received thiouracil, and the thyroid shows no deviation from normal.

Figure X shows a ninth generation tumor line established from a lung nodule.

Figure XI shows a low-power view of a lung metastasis from the ninth generation tumor. Figure XII shows a high-power view of the same metastasis. The cell arrangement is similar to that of the subcutaneous tumor in the same animal.

Figure XIII shows a line four thyroid tumor also in the ninth generation. This tumor was originated from the transplantation of a hyperplastic thyroid. This section shows the sumor grown in a mouse ingesting thiouracil, acidophylic colloid being present in the follicles. Figure XIV shows a subcutaneous ninth generation transplant of the same tumor growing in a mouse without thiouracil. The histological structure of this tumor is essentially similar to that shown in the animal ingesting thiouracil.

A summary of some preliminary studies of the characteristics of four tumor lines (Wollman, Morris, and Green, 1951) in-

FIGURE IX

FIG. IX. Thyroid of a mouse on stock diet bearing a ninth generation transplantable tumor, X 300, line one. Thyroid shows no deviation from normal.

FIGURE X

FIG. X. Transplantable ninth generation thyroid tumor produced from a lung nodule, line one. Note cord-like arrangement of cells and absence of colloid. X 300.

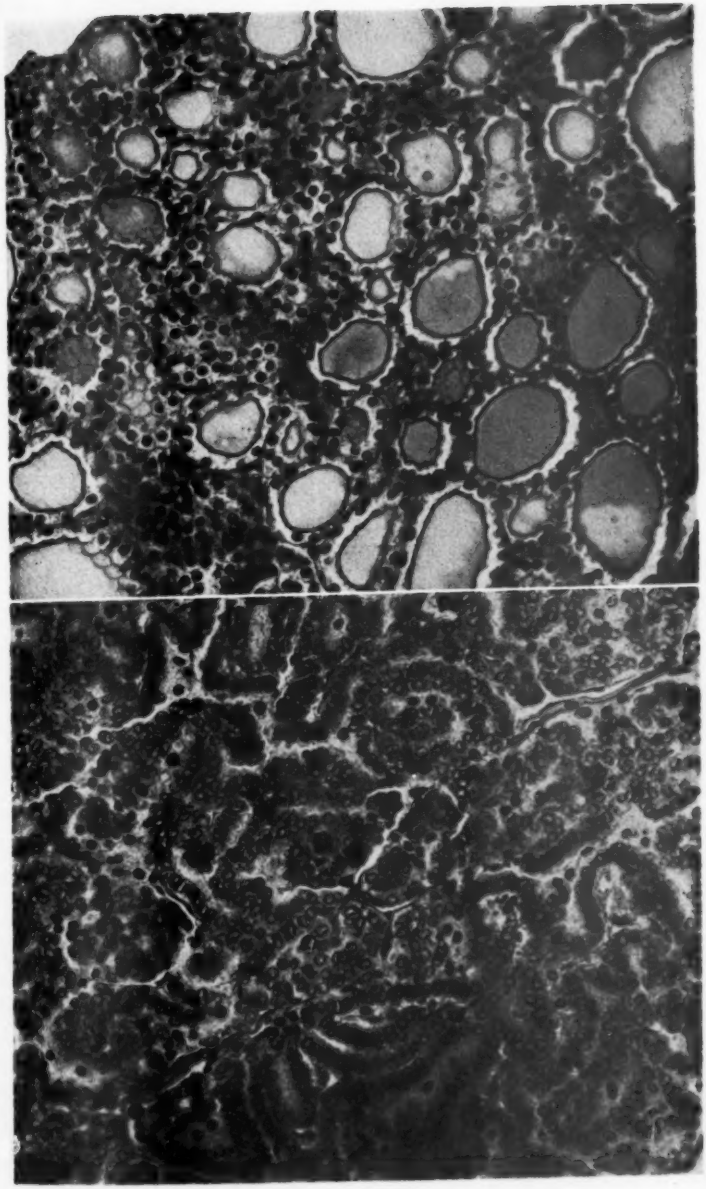


FIGURE XI

FIG. XI. Lung metastasis from ninth generation subcutaneous transplant of thyroid tumor, line one. X 65.

FIGURE XII

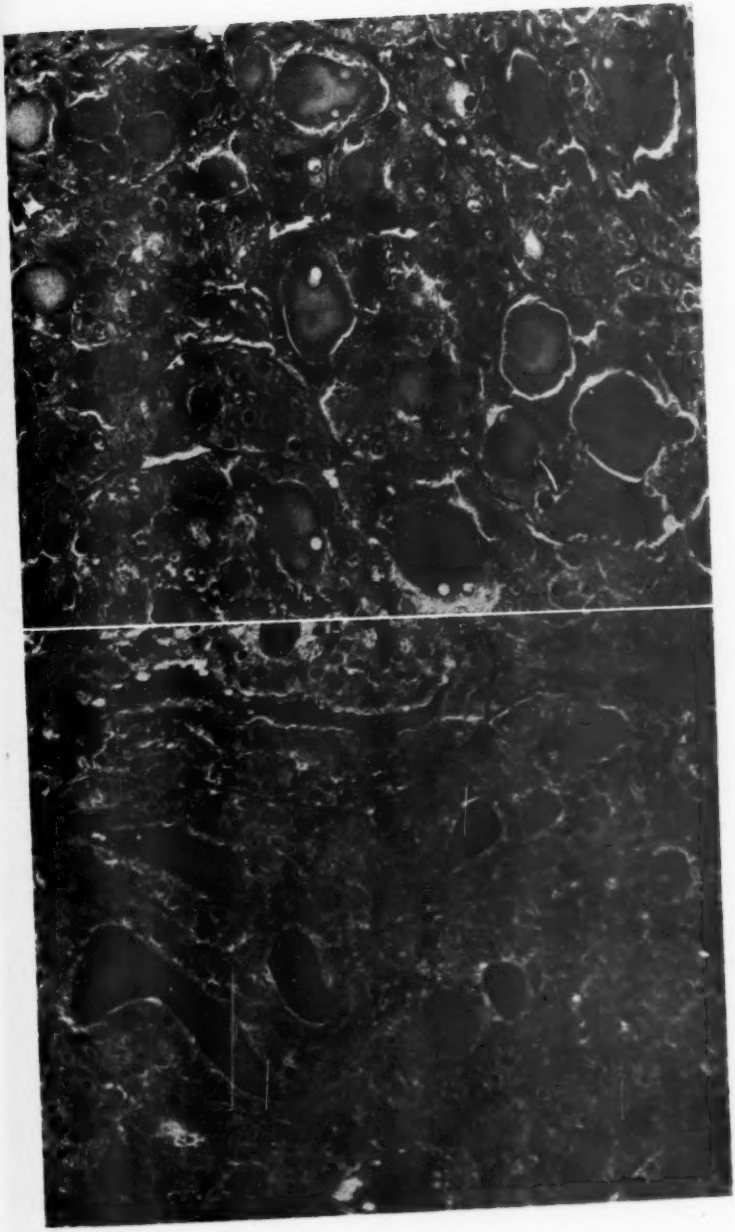
FIG. XII. High-power view of the pulmonary metastis shown in figure XI. Note cell arrangement similar to that of tumor shown in figure X. X 300.

FIGURE XIII

FIG. XIII. Subcutaneous transplant of ninth generation, line four, thyroid tumor grown in a mouse ingesting thiouracil. Note acidophylic colloid present in follicles. X 300.

FIGURE XIV

FIG. XIV. Subcutaneous transplant of ninth generation, line four, thyroid tumor grown in a mouse on stock diet, no thiouracil was ingested. Detailed structure similar to that shown in figure 13. X 300.



vestigated to see if they retain ability to take up I^{131} and convert I^{131} to thyroxine is shown in table 4 (Wollman, Morris, and Green, 1951). It is quite apparent that these four tumor lines are functionally different. The I^{131} concentrating power relative to the thyroid of line one was 1/1300; line 2, 1/164; line 3, 1/10.3; and line 4, 1/2. The tumors would have to be two, ten, 164, or 1,300 times the size of their respective hose thyroids to concentrate the same amount of iodine. Even the most active of these tumors at the time this experiment was carried out was only one-half as active in concentrating I^{131} as the thyroid of its host, and some lines had very slight activity.

TABLE 4

Some Characteristics of Tumors of Thyroid Gland in C₃H Mice. Line four had not yet Become Independent of Thiouracil Ingestion (27)

Tumor	Time on thiouracil*	Growth independent of thiouracil	Histology†	I^{131} concentrating power relative to thyroid gland	Per cent I^{131} picked up in thyroxine of tumor relative to thyroid gland
1	57	Yes	Adenocarcinoma;‡ long tubules; no colloid.	1/1300	...
2	86	Yes	Adenomatous, colloid in tissue space.	1/164	0.14
3	98	Yes	Adenocarcinoma;§ many follicles, variable size; light-staining colloid.	1/10.3	.57
4	98	No	Adenoma; numerous small follicles.	1/2.16	1.1

*Time on thiouracil: Time in days required for most of the tumors to grow to an estimated weight of 100 mg. while hosts were being fed a diet containing thiouracil.
†The histologic characteristics described here are those of the immediate parent tumors from which the present tumors were derived. The histologic evaluation was kindly given by Dr. A. J. Dalton of the National Cancer Institute.
‡Invasion of muscle
§Metastasis to lung.

The table shows that three of the lines possess ability to convert iodide to thyroxine. Eleven to 16 per cent of I^{131} in the thyroid gland was present in the thyroxine fraction at 18 hours while only 2.4 to 12 per cent of the I^{131} in the tumor was estimated as thyroxine. The ratio of the I^{131} fraction, as thyroxine of the tumor to that of the thyroid, varied from one-seventh to that equally as good as the same function in the thyroid of the host.

Has the thyroxine in these tumors been synthesized through the same pathway as occurs in its synthesis by normal thyroids?

A radioautograph of paper strip chromatograms from the thyroids and tumors of mice 25 hours after administration of I^{131} is given in figure XV (Morris and Wagner, 1952). The

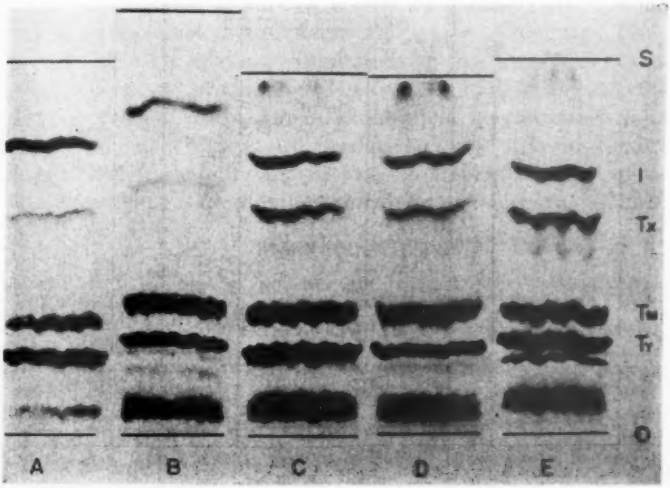


FIG. XV. Radioautograph of paper strip chromatograms of A—thyroids, B—thyroids of tumor-bearing mice, C, D and E—thyroid tumors, O—line of origin, Ty—band for diiodotyrosine, Tm—band for monoiiodotyrosine, Tx—thyroxine band, I—inorganic iodide, S—solvent front.

same bands are present in the sumors as are present in the thyroids of normal mice or thyroids of tumor-bearing mice, *i.e.*, diiodotyrosine, monoiodotyrosine, and thyroxine.

The average uptake of I^{131} in ten tumors from mice ingesting thiouracil was 0.04 per cent per mg. tumor and the average for 21 tumors growing in mice not ingesting thiouracil was 0.047 per cent per mg. tumor as shown in table 5. The uptake per mg. was essentially the same in both groups. The growth of the tumors was 32.2 mg. and 12.2 mg. tumor per week, respectively, a clear indication that thyroid tumor growth was accelerated by ingestion of thiouracil. The increased secretion of TSH seems to be the most likely explanation of this increased tumor growth in this tumor line.

TABLE 5

Summary of I^{131} Uptake in Thyroids and Thyroid Tumors and Rate of Thyroid Tumor Growth in Line Four Tumors in C_3H Mice

Exp. No.	No. Tumors	No. Mice	Thyroids Avg. Wt. mgm.	Thyroids I^{131} Uptake Avg. %	Thyroids I^{131} Uptake Avg. mgm.	Tumor Avg. Wt. mgm.	Tumor I^{131} Uptake Avg. %	Tumor I^{131} Uptake Avg. %/mgm.	Avg. rate of growth mgm./week
				AUTONOMOUS					
I	11	8	2.3*	.84	0.40	368	18.7	.051	...
II	10	10	1.9†	3.4	1.8	160	5.9	.037	...
I + II (Total)	21	18	...	...	...	269	12.6	.047	12.2
				THIOURACIL—DEPENDENT					
I	3	3	15.2	9.8	0.64	619	21.5	.034	...
II	7	7	16.4	13.6	0.83	650	28.9	.044	...
I + II (Total)	10	10	...	...	...	641	26.0	.040	32.2
				NON-TUMOR, NON-THIOURACIL CONTROLS					
		8	2.25	10.8	4.8				

*Average of 4 animals.
†Average of 6 animals.

Summary

Nutritional deficiencies, which effect spontaneous mammary tumor development in mice, appear to act primarily by altering the hormonal secretions of the anterior pituitary and the ovary. Thiouracil ingestion is also believed to inhibit mammary tumor development in mice by way of the pituitary and ovarian secretions similarly to nutritional deficiencies. Thiouracil, however, is believed to cause simultaneously an increased thyrotrophic hormone production. This excess TSH stimulates the thyroid tissue; and if such stimulation is continued long enough, thyroid tumors are produced. These thyroid tumors have a variable affinity for I^{131}, and some can convert iodide into thyroxine by the same intermediates as the thyroid glands.

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MECHANISMS OF ACTION OF CERTAIN AGENTS WITH ANTI-CANCER ACTIVITY

HOWARD E. SKIPPER

When one considers the fact that antagonists of citrovorum factor, a coenzyme involved in nucleic acid synthesis, and several analogs of nucleic acid bases possess the ability to increase the life span of leukemic mice and to inhibit certain transplanted tumors in experimental animals, it is of great interest to study the effects of anti-cancer agents on the metabolism of these important chromosome and cytoplasmic constituents, nucleic acids.

Many present-day workers consider cancer a disease of mutation. Mutability is associated with one biologic substance, nucleoprotein, which is a constituent of two self-duplicating biologic entities, chromosomes and viruses. An important characteristic of all genes (which lie along the chromosome) is nucleoprotein, which is a constituent of two self-duplicating tion in one or more altered forms. Genes appear to contain DNA and to direct the production of specific proteins including biological catalysts, enzymes. Thus, it seems possible that cancer is associated with altered nucleoprotein, which multiplies in its altered form and which influences the production of enzymes and, in turn, the rate at which chemical equilibria are reached in cells.

Boyland (1950) has suggested that carcinogens which damage the chromosome may operate by inactivation of genes, since it seems unlikely that a toxic agent should add something to nuclear material. This suggestion could be fitted into Potter's theory that cancer is a cellular reflection of inadequate production of certain enzymes (resulting from gene inactivation) necessary for continuous degradation of protoplasmic building blocks in normal growth control (1944). We are impressed by the almost exact overlapping of the ultraviolet absorption spectra of nucleic acids and the relative cytotoxicity and mutagenic activity of various wave lengths of ultraviolet light.

The evidence that genes control cellular metabolism by influence on enzymes is now quite convincing. When *E. coli*,

for instance, is treated with ultraviolet light, it is possible to isolate many mutant strains which require an amino-acid, a vitamin, or a purine not necessary for growth of the parent strain (Davis, 1950). This suggests that the U. V. light has changed the structure of certain substances in a given gene (perhaps the DNA-protein) so that this gene cannot reproduce itself. Therefore, the enzyme required for catalyzing a certain chemical reaction is not produced, and the product of this reaction must be supplied in the media for growth.

If there are enzymes which control growth by maintaining a balance with regard to protoplasmic precursors, then one might postulate that in cancer there are enzyme deficiencies caused by damage to certain genes; as a result, the excess building blocks are not degraded, as would be the case in normal cells, but are used for growth.

In general, we assume that growth is dependent on an increase in protein and nucleic acid. The presence of RNA in the sites of protein synthesis has led many workers to believe that this nucleic acid is involved in protein synthesis in the cytoplasmic microsomes. Haurowitz (1950) points out that globular protein molecules with diameters of 30–100 Å cannot be reproduced as three-dimensional corpuscles (assuming a template mechanism), because intermolecular forces operate only over distances of less than 3–5 Å. Therefore, it might be assumed that the protein molecule in the phase of reproduction or formation is in a monomolecular film (the unfolded state). This author postulates that nucleic acids may function in protein synthesis by formation of an insoluble rigid layer of the protein template. Or as stated by Haurowitz, "nucleic acid present in the site of protein synthesis forms an insoluble nucleoprotein compound with the template protein and thus is responsible for the rigidity of the template layer and for its insolubility and persistence." The continuous synthesis of protein could then be the result of adsorption of amino acids on the protein template and later folding of the protein formed, converting it to the globular state; this allows new amino acids to be absorbed to the surface of the template.

This sort of mental gymnastics might be oversimplified by a drawing such as is present as figure I.

Here DNA is shown as a stretcher for the protein template in a gene with a replicate of the gene protein, an enzyme, being formed, which will fold and become an enzyme molecule. Such reasoning would explain in a general sort of way why carcinogens which affect DNA are mutagenic and result in enzyme deficiencies and why inhibitors of nucleic acid synthesis (DNA and RNA) are such profound inhibitors of growth. The question of why certain anti-cancer agents preferentially damage cancer cells is still an unanswered one. It seems possible that this specificity may be based on quantitative differentials, since these agents usually damage the mitotically active intestinal epithelium.

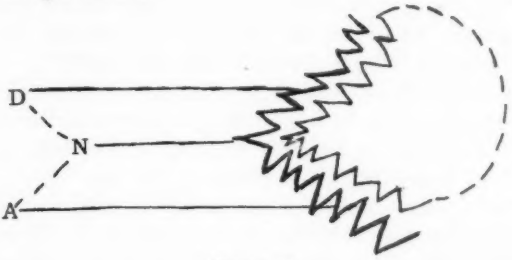
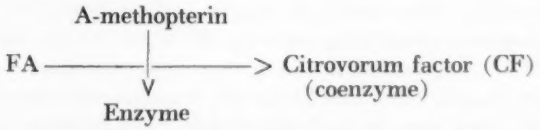


FIGURE I

As has been mentioned by Dr. Borquist, 4-aminopteroylglutamic acid (aminopterin) and 4-amino-N^{10}-methylpteroylglutamic acid (A-methopterin) are profound inhibitors of bacteria and certain neoplastic cells.

Borrowing from general schemes suggested by Shive (1950), it appears that folic acid or citrovorum factor antagonists may provide anti-leukemic action by competition with the enzymes (s) involved in conversion of folic acid to citrovorum factor (Nichol and Welch, 1951).



Then, because of the reduction in amounts of CF coenzyme, a number of reactions involving transfer of formate may be inhibited.

Formate —CF–Enz A→ Thymidine

Formate —CF–Enz B→ Purines nucleotides

Formate —CF–Enz C→ Serine

Formate —CF–Enz D→ Methionine

In bacteria, Broquist and his associates have shown that thymidine non-competitively prevents the growth inhibition of aminopterin (1950), while citrovorum factor competitively reverses this antagonist. Burchenal (8) has shown that folic acid and citrovorum factor competitively prevent the anti-leukemic action of 4-aminopteroylglutamic acids.

We have but recently been able to isolate sufficient thymidine to begin consideration of inhibition analysis with this material and A-methopterin in leukemic animals. It has been possible. however, to show with Ak-4 leukemia in mice that purines, pyrimidines, serine, methionine, formate, and ascorbic acid fail to prevent the anti-leukemic action of A-methopterin. DNA and B_{12} as well as combinations of guanylic acid, adenylic acid, and cytidylic acid partially prevented the anti-leukemic action of 4-aminopteroylglutamic acids. It should be most interesting to study thymidine in this respect along with combinations of purine nucleotides and thymidine.

These data suggest that it may well be the inhibition of nucleic acid synthesis which accounts for the anti-cancer activity of citrovorum factor antagonists. The answer, as to which formate transformation in nucleic and synthesis within the cancer cell is the limiting one, must await further study.

Dr. Brown is going to discuss metabolic and chemotherapeutic studies with purine derivatives, so it will not be necessary for me to spend time covering the literature in this field. I would like to mention, however, that we have obtained strong evidence that 2, 6-diaminopurine and 8-azaguanine are "fixed" in small quantities, as such, in nucleic acids of mice; we are now trying to devise experiments which might answer the question of whether this incorporation of "fraudulent" purines has to do with the action of these temporary anti-cancer agents.

In the case of diaminopurine, there is evidence that this purine may be an intermediate in the biosynthesis of guanylic acid. If this can be proven, then it would appear that diaminopurine may be acting in neoplastic cells as an inhibitory metabolite when the concentration is high enough.

Referring briefly to the template mechanism of protein reproduction and the possible role of nucleic acids in this process, it is conceivable that a fraudulent purine could seriously disturb the alignment of the nucleic acid-protein template, resulting in preferential damage to cells with quantitatively greater demand for protein synthesis (growth). The fact that guanosine or quanylic acid, but not purines, will partially prevent the anti-cancer activity of 8-azaguanine (Gellhorn) suggests that this purine-like compound may be converted into a ribose derivative; as such, it may compete with normal nucleotides.

Nitrogen mustard we know as a very active alkylating agent which preferentially inhibits certain types of neoplastic cells. Sulfur mustard causes mutations in Drosophila (Auerbach and Robson, 1946) and combines with proteins and with the NH_2 or the H_2PO_3 groups of nucleic acids (Elmore, *et al.*, 1948). Dr. Wheeler of our group has shown conclusively that nitrogen mustard will react with guanine or DNA *in vitro* and that HN_2-purine reaction products can be isolated (by paper or ion-exchange chromatography) with characteristic absorption spectra.

Another factor which might lead one to consider that anti-cancer agents in general may be affecting some phase of nucleoprotein metabolism is the fascinating recent observation that certain viruses (self-duplicating nucleoprotein particles) are particularly damaging to experimental tumors.

In summary, let us consider certain evidence (experimental and theoretical) which might be fitted together to make a general picture regarding the mode of action of certain classes of anti-cancer agents.

Cancer seems to be a disease of mutation originating from cells in which gene (nucleoprotein) changes have occurred. Since genes control production of specific enzymes, we might assume that cancer cells have quantitatively or qualitatively different enzyme or cell catalyst patterns from those found

in normal cells. Since enzymes effect only rates of chemical reactions, we could expect cancer cells to differ from normal cells basically with regard to chemical equilibria.

There is evidence that several anti-cancer agents, *i.e.*, citrovorum factor antagonists, purine antagonists, radiation, and nitrogen mustard, may be acting on cellular nucleoprotein metabolism.

If one assumes the template mechanism of gene reproduction and specific enzyme formation, then one might postulate that the cytotoxic action of A-methopterin is primarily caused by inhibition of coenzymes having to do with *de novo* nucleic acid synthesis; thus, formation of an integral portion of the template is prevented. The purine antagonists might act by derangement of the nucleic acid fraction of the template. Nitrogen mustard and its companion chemotherapeutic agent, triethylene melamine, may provide cytotoxic effects by alkylation of nucleic acid moieties. It is not inconceivable that radiation provides anti-cancer effects directed at the nucleoprotein template.

No suitable explanation for the slight specificity of action of anti-cancer agents on neoplastic cells has yet been provided, except for the assumption of specificity caused by quantitative rate differentials. It is possible that production of proper templates (gene substances) is the rate-limiting factor in cancer cells geared to rapid anabolic processes (growth). An equal depression of template formate and, in turn, certain enzyme production is more detrimental to the cancer cell than the normal cell.

It can be expected that work by scientists such as are assembled here today will do much to unravel the existing questions on the mode of action of anti-cancer agents. Such knowledge will be of tremendous importance to an understanding of the disease, its origin, its intermediary metabolic deviations from the normal, and finally, we hope, its control.

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CANCER CHEMOTHERAPY EXPERIMENTS WITH PLANT EXTRACTS

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The problem of finding a chemical agent which will completely inhibit the growth of or destroy tumor tissue without causing undue disturbance to nontumor tissue is one which numerous investigators have demonstrated to be most difficult. Helen Dyer (1949) in her *An Index of Tumor Chemotherapy*, lists 5,031 chemical substances used in chemotherapy tests. The tempo of the work in this field appears to have increased since the 1949 report so that the number of tested compounds is much larger at the present time. In spite of all this effort, however, no one has claimed to have discovered a practical chemotherapy for any of the numerous types of malignant growths.

The discovery that certain compounds occurring naturally in fungi and other plant growths can be used to control some of the diseases associated with pathogenic bacteria has emphasized the fact that plant and animal organisms contain a practically limitless array of chemical compounds. Plants in particular have long been a source of chemical substances with unusual and often specific effects on animal organisms. Some of our most effective therapeutic agents are of this origin.

A survey of the literature on tumor chemotherapy discloses that very little work has been done with plant extracts. Some plant products, such as colchicine and podophyllotoxin, have been and still are the subject of investigation, but very few systematic surveys of the possibilities of the plant world in this regard have been undertaken. Such work as has been done with plants has usually been carried out in such a manner as to conserve only the chemically more stable materials. The procedures generally employed would not have preserved many

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of the most potent antibacterial agents in use at the present time.

Recently a project was set up at The University of Texas, the objective of which was to screen various plant extracts for compounds with a possible inhibitory action on the growth of tumor tissue. Plants known to be toxic when eaten by animals were selected for the initial work. The purpose of this study was to explore the reservoir of ready-made compounds contained in plant tissue by a type of processing that would effect a minimum degree of disturbance to the chemistry of the extracts before the material was tested for its effects on the growth of tumor tissue. It was realized that while probably only a small fraction of the compound present in the living plant cells can be preserved in extracts long enough for testing, nevertheless, there should still be available a large variety of biologically reactive substances.

During the eleven years cancer research has been in progress at the University Biochemical Institute, methods have been developed for rapidly evaluating the tumor chemotherapeutic possibilities of a particular substance. Egg-cultivated tumor tissue has been used extensively in these studies. A reasonably good 24-hour test has been developed which greatly shortens the time required to screen through compounds and extracts. The use of yolk-sac-cultivated tumors makes it possible to compare the reactions of neoplastic and rapidly proliferating non-neoplastic tissue to the test compound. The relationship of the yolk-sac tumors and the host embryo are such that, in the early stages, they maintain vigorous and independent growth and at the same time share a common blood stream and a common environment (Taylor and Carmichael, 1948; Taylor, 1950; Taylor and Carmichael, 1951; Taylor *et al.*, 1951).

Parallel and supplementary to the use of egg-cultivated tumor tissue, mouse-grown tumors have been used in large numbers. Hence, the techniques for testing the effect of the plant extracts on tumor growth were fairly well standardized.

A brief consideration of the methods used and results obtained with a few species of plants will demonstrate something of the possibilities of this approach for cancer therapy.

Experimental Procedures

Preparation of extracts. The plant material was processed as soon as possible after collection. Roots, leaves, stems, and berries were sometimes extracted as a conglomerate; but usually, these parts were treated separately. The various manipulations associated with making the extracts were carried out at as low a temperature as practical in order to minimize chemical changes, and the extracts were tested for their biological effects as soon as possible after preparation.

For the first tests of a plant, the following extracts were made: aqueous, pH 7.0; aqueous, pH 6.6; aqueous, pH 7.4; hot aqueous, pH 7.0; acetone; and absolute alcohol. If the results with these extracts warranted further work, efforts were made to concentrate the active principle.

In the earlier work, the aqueous extracts were tested without checking the amount of dry material in solution, since if negative results were obtained this extra work could be omitted. More experience with the project has demonstrated that a plant may yield an extract which has a considerable inhibitory effect on tissue growth only at a certain stage in its development or at one season of the year. In the instance of rain lily bulbs, it was found that the specific gravity of the aqueous acid extracts differed in the spring from what it was in the winter. Accordingly, it has been found desirable first to make extracts of the fresh plant material, and second to reduce these to the contained solid material by low temperature high vacuum dessication. This results in a relatively stable material which can be redissolved at the time it is tested for its effect on the growth of tumor tissue. Further, in order to preserve substantial quantities of a plant at a particular season, the parts used for the extract are lyophilized and stored at deep freeze temperatures. The various extracts are then prepared from this lyophilized material.

It has been noted that the biological activity of plant extracts made at low temperatures diminishes with time. An effect of a solution on tumor or embryo growth may be considerably less after standing a few days at ordinary refrigerator temperature; and even when kept at much lower temperatures, there may be some deterioration.

It is difficult to maintain sterility in extracts prepared by the methods described above. When plant material is such that the outer portion can be removed, it is possible to have sterile conditions at every step in the process of extracting. In most instances, sterility of the extract was achieved by filtration.

Methods of testing. The extracts were first checked for sterility and then introduced into nontumor embryonated eggs for toxicity evaluation. Injections were made at various concentrations over the blood vessels of the area vasculosa of the chick embryos on the eleventh day of incubation. In this way, data were obtained which indicated the level that could be used with tumor-bearing eggs. Also, the information gained in this manner applied generally to the dosages tolerated by mice.

Tumor-bearing eggs were prepared as previously reported (Taylor, *et al.*, 1942; Taylor, *et al.*, 1943; Taylor, *et al.*, 1948). The method essentially involves yolk sac inoculation through the air sac of embryonated eggs on the fourth day of incubation with a saline suspension of dispersed tumor tissue. Eggs were implanted with a C_3H mouse mammary carcinoma which grows with relative uniformity in every inoculated egg. This tumor implanted in the eggs by the yolk sac method attains a size of 0.2 gm. eight days after the eggs are inoculated or on the twelfth day of incubation. Between the twelfth and thirteen days, the tumor doubles in size. The various extracts were introduced over the area vasculosa of twelve-day tumor bearing eggs by a method described in detail elsewhere (Taylor, 1950). The method of introducing the test compound into the egg which was used in these experiments insures absorption into the chick blood system. In a recent paper Galinsky (1950) demonstrated that radioactive phosphorus injected by this technique over the chorioallantoic membranes and area vasculosa was taken up by the blood stream and distributed to the chick embryo, yolk sac tumor, and other parts of the egg. The rate of absorption naturally varies with the type of material injected. In the present investigation, the best results were obtained when absorption of the inoculum was made to proceed more slowly by the use of cottonseed oil in the suspending medium (two parts oil and one part medium). Embryos and tumors were harvested on the thirteenth day, and the growth of em-

bryos and tumors in the experimental groups was compared with that which occurred in the controls.

A fast-growing transplant sarcoma was used for testing the effect of the various extracts on mouse-cultivated tumors. For a preliminary evaluation, the test material was introduced into a saline suspension of dispersed tumor tissue and incubated for one hour at 37° C. The control tubes contained only the saline suspension of cells. The effect of an extract was evaluated by the growth of subdermal implants of the tumor suspension in the host mouse as compared with the growth of the control implants. This appears to be a simple way of eliminating the great majority of ineffective extracts from further consideration. It would seem that if a particular material has any tumor inhibiting capacity, it should become evident under these conditions.

To date, most of the testing program which is still in a preliminary stage has been concerned with the effect of the various extracts on the tumor and embryo of tumor-bearing eggs.

Results

About 250 extracts have been prepared and tested to date. As is usual with such studies, most of them have had only slight effect on tumor or embryo growth. Some have proved to be so toxic that the inhibiting effect on the growth of the tumor has little significance. Quite a number of extracts have inhibited the growth of both tumor and embryo of tumor-bearing eggs. In some instances, tumor growth seems to have been inhibited with no effect on the embryo.

The variability in biological activity that can be expected from plant extracts and something of the possibilities latent in this source of chemical substances can best be seen by a consideration of the results with particular plants. First, however, in order to demonstrate the validity of using egg-cultivated tumors in these tests, the results of a series of trials with an analog of folic acid, 4-aminopteroylglutamic acid (aminopterin); an analog of riboflavin, isoriboflavin; podophyllotoxin; and cortisone acetate are given in table 1.[1] It will be noted

[1] Aminopterin supplied by Lederle Laboratories, isoriboflavin by Dr. Karl Folkers of Merck & Co., and podophyllotoxin by S. B. Penick & Co.

TABLE 1

The Effect of Aminopterin, Cortisone, Podophyllotoxin, and Isoriboflavin on the Growth of Tumor and Embryo of Tumor-Bearing Eggs. 24 Hour Test Period.

Material	No. exps.	No. eggs	Dosage (mg.)	Survival, control =100	Avg. tumor wt. end of test period		Avg. embryo wt. end of test period		Growth of exp. groups for test period, control =100	
					Exp.	Control	Exp.	Control	Tumor	Embryo
Aminopterin	5	128	0.1	86	.27±.03	.47±.04	4.30±.08	4.54±.09	13	81
Cortisone	5	160	0.1–1.5	100	.57±.04	.72±.05	3.80±.12	4.33±.07	57	50
Podophyllotoxin	5	150	0.002	100	.49±.05	.67±.08	4.12±.12	4.36±.13	34	77
Isoriboflavin	3	92	0.05	89	.50±.03	.66±.03	4.03±.11	4.18±.16	41	85

that the well-known growth-inhibiting capacity of these compounds was demonstrated by the method used. Further, that these compounds have no specific effect on neoplastic tissue was manifested by the fact that the chick embryos as well as the tumors were inhibited in growth.

Rain lily. Extracts from two genera, *Zephyranthes texana Herb.* and *Cooperia pedunculata Herb.*, were used. The two species appear to be equivalent from the standpoint of their inhibiting effect on egg-cultivated tumors.

Table 2 contains a résumé of the most effective rain lily extracts. It will be noted that the April acetone and acid aqueous extracts almost completely inhibited the growth of egg-cultivated tumors for the period of the test without affecting the associated chick embryos. The extracts were superior in this respect to aminopterin which has about the same inhibiting effect on the growth of the tumor, but affects the chick embryo also.

The influence of the season on plant extracts is shown in the data given in table 2. There was a marked difference in the tumor growth-inhibiting activity of comparable extracts made from the plant in April and September. The September extracts have much less effect on the tumor, but the embryo continued to be unaffected as to growth. Further work is being done on isolated constituents of the extracts.

Enough tests were completed for all the items of table 2 to give good statistical validity for the differences recorded between control and experimental groups.

Other types of extracts of the rain lily were relatively ineffective.

Very little work with mouse-cultivated tumors has been done with this plant as yet. An acetone September extract has prevented the growth of transplants of a sarcoma when incubated in the tumor suspension for one hour at 37° C. before implantation in the mouse.

Buffalo bur (Solanum rostratum Dunal.) and Milkweed (Asclepias decumbens L.). These two plants have given ex-

TABLE 2

The Effect of Extracts of Rain Lily Bulbs on the Growth of Tumor and Embryo of Tumor-Bearing Eggs. 24 Hour Test Period

Type of extract	No. expe.	No. eggs	Dosage	Survival, control =100	Avg. tumor wt. end of test period		Avg. embryo wt. end of test period		Growth of exp. groups for test period, control =100	
					Exp.	Control	Exp.	Control	Tumor	Embryo
Cooperia, aqueous, pH 6.6 April	4	112	0.1 cc.	100	.28±.05	.45±.06	4.75±.13	4.73±.09	9	101
Cooperia, acetone, April	3	80	0.5 mg.	100	.28±.01	.46±.07	4.81±.13	4.72±.12	5	101
Cooperia, aqueous, pH 6.6 September	4	106	0.1 cc.	100	.39±.09	.48±.02	4.71±.41	4.73±.40	54	97
Zephyranthes, aqueous, pH 6.6 September	5	130	0.1 cc.	100	.36±.06	.44±.07	4.42±.16	4.33±.33	56	106

tracts, table 3, which indicate the general tendency for whatever growth inhibitors there are in an extract to affect both embryo and tumor. The aqueous extract of the buffalo bur does appear to be more potent in its effect on the tumor as compared with the embryo.

TABLE 3

The Effect of Various Plant Extracts on the Growth of Tumor and Embryo of Tumor-Bearing Eggs. 24 Hour Test Period

Type of extract	No. exps.	No. eggs	Dosage	Survival, control =100	Avg. growth for test period control=100 Tumor	Embryo
			Buffalo bur			
Aqueous, leaves & stems	1	30	0.1 cc.	100	26	87
Acetone, leaves & stems	2	40	0.1 cc.	100	74	102
			Milkweed			
Aqueous, pH 7.0, whole plant except root	1	28	0.1 cc.	100	91	82
Aqueous, pH 6.6, whole plant except root	1	32	0.1 cc.	100	81	87
Aqueous, pH 7.5, whole plant except root	1	26	0.01 cc.	100	120	87
			Castor Bean			
Aqueous, seeds	4	64	0.004 cc.	74	13	90
Aqueous, seeds	1	32	0.035 mcg.	100	78	107
Aqueous, seeds	1	32	0.07 mcg.	100	38	95
Aqueous, seeds	1	16	0.7 mcg.	100	5	76
Acetone, seeds	1	16	1.0 mg.	100	96	114
			Mistletoe			
Aqueous, pH 7.0, berries, stems & leaves	5	110	0.1 cc.	100	60	90
Aqueous, pH 6.6, berries, stems & leaves	2	36	1.0 mg.	97	80	57
Aqueous, pH 7.4, fresh plant	1	16	1.0 mg.	100	118	95
Acetone, leaves & stems	1	30	1.0 mg.	87	44	82
Alcohol, dried plant	2	40	1.0 mg.	91	116	100

TABLE 3—continued

The Effect of Various Plant Extracts on the Growth of Tumor and Embryo of Tumor-Bearing Eggs. 24 Hour Test Period

Type of extract	No. exps.	No. eggs	Dosage	Survival, control =100	Avg. growth for test period control=100 Tumor	Embryo
Poison Ivy						
Aqueous, pH 7.5, leaves	6	150	0.1 cc.	100	72	100
Aqueous, pH 6.6, leaves	1	26	0.1 cc.	100	112	118
Hot aqueous, leaves	2	50	0.5–1.0 mg.	100	53	91
Hot aqueous, leaves & stems	1	30	0.02 cc.	100	15	80
Acetone, leaves & stems	2	38	0.02 cc.	100	148	66
Alcohol, leaves	1	16	0.1 cc.	100	47	73
Snow-on-the-mountain						
Aqueous, 7.0, whole plant	1	28	0.2 cc.	100	55	91
Aqueous, pH 7.6, whole plant	1	30	0.1 cc.	100	60	59
Acetone, whole plant	1	30	0.1 cc.	100	78	59
Alcohol, whole plant	1	26	0.1 cc.	100	79	115
Yucca						
Aqueous, roots	3	50	0.1 mg.	100	27	80
Acetone, roots	8	160	0.1 mg.	100	54	93
Acetone, new leaves	2	30	0.1 cc.	100	87	87
Alcohol, roots	2	34	1.0 mg.	100	13	100

Castor bean (Ricinus communis L.). Table 3 summarizes the work with extracts of seeds of this plant. It was discovered, as others have reported before, that an aqueous extract of castor bean seeds contain ricin, an extremely toxic compound which is of little practical value from the standpoint of its effect on tumor growth. As table 3 records, less than 0.1 microgram of the material obtained by lyophilizing an aqueous extract of crushed seeds inhibited tumor growth about 60 per cent. The embryo appears to have been relatively little affected. More prolonged tests revealed that the full toxicity of the castor bean extract requires more than 24 hours for its developbment. A dosage level of 0.31 micrograms per kilogram of body weight of purified ricin has been reported to be lethal in 18 hours for rabbits (Osborne, *et al.*, 1905).

American mistletoe (Arceuthobium vaginatum Eichler). The results obtained with this plant, table 3, give little promise as far as egg-cultivated tumors are concerned. It will be noted that various extracts differed in their effect on the growth. Several of the extracts affected the survival of the eggs, indicating some toxicity.

Experiments with mice have shown that the berries of this plant contain a highly toxic fraction. Also, it has been observed that extracts of the berries at relatively nontoxic concentrations in one-hour-incubated saline suspensions of a sarcoma were associated with complete inhibition of growth when implanted into the mouse.

Poison ivy (Rhus toxicodendron L.). The results with this plant are summarized in table 3. The work with poison ivy was complicated by the seasonal variation encountered in extracts and the apparent presence of growth inhibitors and accelerators. A number of experiments have been carried out with nontumor eggs which indicate the presence of a growth stimulator in hot aqueous extracts of spring collections of leaves. At the same time, as the table shows, a growth inhibitor for both tumor and embryo was present in hot aqueous extracts of leaves and stems of fall and winter plants.

Snow-on-the-mountain (Euphorbia marginata Pursh). Extracts of this plant tended to be inhibiting in their effect on the growth of tumor and embryo of tumor-bearing eggs. It is worthy of note that an acetone extract affected embryo growth more than that of the associated tumor. This is rarely encountered, since the growth rate of the chick embryo from the twenfth to the thirteenth days of incubation is not quite half the rate of that of the yolk sac tumor, it is less vulnerable to the growth inhibitor.

Yucca (Yucca arkansana Trel.). The root portion of this plant has given some indication, table 3, that there is a growth inhibitor in both the aqueous and alcohol extracts which affects tumor more than embryo tissue.

Acid, basic, or neutral aqueous extracts incubated with saline suspensions of mouse sarcom were associated with complete or nearly complete inhibitions of growth of the implant in mice.

Table 4 summarizes the most promising results obtained with plant extracts and includes the data given in table 1 for comparison. These data show that from five different plants, extracts have been obtained which compare favorably with aminopterin from the standpoint of their inhibiting effect on the growth of egg-cultivated mouse mammary carcinoma; and extracts from two of the plants, yucca and rain lily, accomplished this without affecting embryo growth.

TABLE 4

The Effect of Various Plant Extracts and other Materials on the Growth of Tumor and Embryo of Tumor-Bearing Eggs. 24 Hour Test Period

Material	Growth of exp. groups for test period, control = 100	
	Tumor	Embryo
Aminopterin	13	81
Podophyllotoxin	34	77
Isoriboflavin	41	85
Mistletoe	44	82
Buffalo Bur	26	87
Yucca, aqueous, roots	27	80
Yucca, alcohol, roots	13	100
Poison Ivy, hot aqueous, leaves & stems	15	80
Rain Lily, aqueous, bulbs	9	101
Rain Lily, acetone, bulbs	5	101

Discussion

The results to date on this project do not warrant any special emphasis. However, the data indicate something of the possibilities of this approach in cancer chemotherapy studies. In past years, many hundreds of compounds have been tested in The University of Texas cancer research laboratories and nothing more promising has come out of these extensive researches than the best of the plant extracts.

Plants, in their long evolutionary history, have developed forms as diverse as oaks and fungi; and variety in form is paralleled by variety in biochemistry. Here is a storehouse of chemical compounds which, even by the rigorous methods of extraction used in the past, has yielded some of man's most potent medicines. This abundance of chemical substances would seem to merit extensive investigation by workers in the field of cancer control. Research programs which would be in a position to screen through thousands of extracts and to follow

up promising leads with chemical isolation and purification procedures should make a real contribution to the solution of the problem of cancer chemotherapy.

Summary

Plant extracts obtained from fresh material processed at low temperatures were tested for their effect on the growth of the embryo and tumor of tumor-bearing eggs. More than 250 extracts have been prepared and tested to date.

Data on the results obtained in this type of test with aminopterin, cortisone acetate, isoriboflavin, and podophyllotoxin are included for comparison. An aqueous extract of buffalo bur, an aqueous extract of yucca roots, and a hot aqueous extract of leaves and stems of poison ivy inhibited tumor and embryo growth to about the same degree as aminopterin. An alcohol extract of yucca roots and acid and acetone extracts of April rain lily bulbs inhibited tumor growth in the aminopterin range without affecting the growth of the embryo.

It is suggested that extensive investigation of the great abundance of untested chemical substances present in plants should make a contribution to the solution of the problem of cancer chemotherapy.

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EFFECT OF THYROXIN UPON GROWTH AND OXYGEN UPTAKE OF *TETRAHYMENA GELEII* (Y)

W. J. Wingo and L. E. Cameron*

The effect of thyroid gland, thyroid extracts, and thyroxin upon growth, metamorphosis, and metabolism of a variety of organisms has been extensively studied. It is generally agreed that these substances will accelerate the metamorphosis of amphibian larvae, and that they are effective at very low concentrations (Gudernatsch, 1913; Romeis, 1922; and Allen, 1932). The oxygen consumption of such prematurely metamorphosing larvae has been reported to be increased (Groebbels, 1922; and Mehes and Berde, 1947).

Physiological effects of substances with thyroid-hormone action upon invertebrates have also been reported. Thus, thyradine increases the oxygen consumption of silkworm larvae (Kurome, 1943); and thyroxin accelerates the rate of eye formation in reconstituting pieces of the flatworm, *Phagocata gracilis* (Weimer, Phillips, and Anderson, 1938). Goldsmith (1949) and Hanstram (1939) have reviewed the effects of thyroid (or thyroxin) administration on various invertebrates, and have found that the state of knowledge in this connection is unsatisfactory, largely because of poorly controlled experiments.

A number of investigators have studied the effects of these agents upon the rate of multiplication of protozoa; the results reported are not consistent. Goldsmith (1949) has summarized these results. In general, the administration of whole thyroid gland substance or crude extracts of thyroid gland appeared to stimulate cell division, while thyroxin either had no effect on or diminished the rate of reproduction. Since these early studies were not done under aseptic conditions, it is quite possible that the stimulatory effects of thyroid gland were caused by its utilization as food for the bacteria, which were in turn the food of the protozoa; the stimulatory effect on protozoa may have been the result of nutritional conditions.

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On the other hand, the few studies on the metabolic as opposed to the reproductive effects of thyroxin upon protozoa have been in agreement, regarding its stimulatory action. Thus, Torrey, Riddle, and Brodie (1925) report a marked increase in the rate of pulsation of the contractile vacuoles of *Paramecium* on thyroxin treatment, and Leichsenring (1925) found that the rate of oxygen consumption of *P. caudatum* was increased by exposure of the organisms to thyroid substance. In all these studies, however, the protozoa were not in bacteria-free culture, so that the results cannot be regarded as other than indicative.

Since the routine culture of *Tetrahymena geleii* in bacteria-free condition is now relatively easy, it occurred to the writers to re-examine the question of the growth and oxygen uptake of protozoa as affected by thyroxin.

Experimental

The (Yellowstone) strain of *Tetrahymena geleii*[1] was chosen for this study, because its ability to grow at elevated temperatures permits its cultivation without recourse to cooling baths in a laboratory which is not air conditioned. All cultures, including stocks, were incubated at 36.9° C.; stock cultures were maintained in medium corresponding to that used for "control" cultures, and transferred at least once a week. Stock transfers and inoculations were made using an inoculating spiral (Wingo, 1950). Slant culture tubes (made with the control medium with 2 per cent agar added) were inoculated from each stock culture before it was discarded and incubated and examined to detect bacterial contamination. If contamination was suspected in an experimental culture, incubation was continued for a day, or longer, and the medium examined microscopically for bacteria after Gram staining.

For the preliminary studies of the effect of thyroxin concentration on reproduction of *T. geleii*, the protozoa were cultured in 250 ml. Erlenmeyer flasks, each containing 50 ml. of medium. The medium employed was the Proteose-Peptone-

[1]Isolated from the water of a hot stream in the Yellowstone National Park by Doctor Austin Phelps, who kindly supplied stock cultures for our use.

mineral medium of Phelps (1936). Thirty ml. of medium was dispensed into each flask, and the desired quantity of thyroxin for each flask was weighed into a small beaker, where it was dissolved in 5 ml. of medium with the aid of sodium hydroxide solution. The solution of thyroxin in medium was added to its flask, and the beaker was rinsed into the flask with 15 ml. of medium. The pH of the medium in each flask was adjusted to 6.3, and the flasks were stoppered with cotton stoppers provided with sampling siphons and sterilized by autoclaving at 15 lbs. pressure for 20 minutes. The flasks were inoculated and incubated as has been described. Periodic counts of the protozoa present were made in a Segewick-Rafter counting chamber, used in connection with a microscope fitted with a calibrated Whipple eyepiece micrometer.

Since in past experiments of this type, a considerable change in pH of the medium occurred as the protozoa grew, the medium was modified by the inclusion of Na_2HPO_4 at 0.05M concentration; the pH was adjusted to 7.3 with hydrochloric acid. (A check of the pH-growth relation had indicated that a pH of 7.3 was near optimal and that the buffering considerably reduced the pH change consequent to protozoan growth.) Repetition of the experiments described in the preceding paragraph using the buffered medium gave substantially the same results as had been obtained before.

For the study of growth and oxygen uptake of protozoa grown in the presence of thyroxin, parallel cultures were grown under aeration both with and without the addition of thyroxin to the medium. For each experiment, a double-strength medium was prepared from concentrated mineral and Proteose-Peptone stock solutions, which were kept in frozen storage. To control foaming in the cultures, 0.2 ml. of oleic acid were added to each liter of double-strength medium. Thyroxin was weighed out in the desired quantity, dissolved in a little water with the aid of sodium hydroxide solution, and added to a volume of the double-strength medium equal to one-half the final volume of thyroxine-containing medium desired. Disodium hydrogen phosphate was added in quantity sufficient to make the concentration 0.05 M after final dilution, and the medium was diluted almost to the final volume. The pH was adjusted

to 7.3 by the addition of hydrochloric acid, and the medium was made up to its final volume. The control medium was made up in the same way with the omission only of thyroxin (a quantity of sodium hydroxide equal to that used to dissolve the thyroxin was added at the same stage in the preparation).

The control and thyroxin media were dispensed in flasks fitted with sealed-in aeration tubes, which were plugged with cotton to prevent introduction of bacteria with the air stream, with spouts for sampling, and with ground-glass-stoppered inoculating ports. Air was passed through each culture at the rate of several bubbles per second. Periodic counts were made as before, and when the number of organisms per milliliter had reached sufficiently high values so that the oxygen uptake of two-milliliter samples of the cultures could be measured, such measurements were made by the use of the ordinary Warburg manometer.

The first set of oxygen-uptake experiments showed that the shaking necessary for the measurements caused the rupture of a considerable portion of the cells. The concentration of calcium in the medium was increased to correct this condition; it was found by trial that a calcium concentration five times that specified by Phelps had no significant effect on growth and prevented almost all the breaking-up of cells which occurred in the medium as first used. In subsequent experiments, therefore, enough calcium to raise the concentration to five times the original was added as a calcium chloride solution during the preparation of the medium from the double-strength stock medium.

Results

The results obtained in the experiments done to determine the effect of thyroxin upon the multiplication of *T. geleii* (Y) are presented in figure I, which is characteristic of the experiments done both in the unbuffered and in the buffered Phenps' medium. At the concentrations employed, no stimulation of cell division was observed; and even at the 20 mg./l level, a pronounced lag phase was encountered. At the higher concentrations, it is not certain whether any multiplication occurred, as the counting method employed has a large probable error

at these levels of population. However, in all the experiments of this character, at the larger concentrations of thyroxin a similar very slow increase in population was indicated by the data; this may represent a very prolonged recovery from a lag phase.

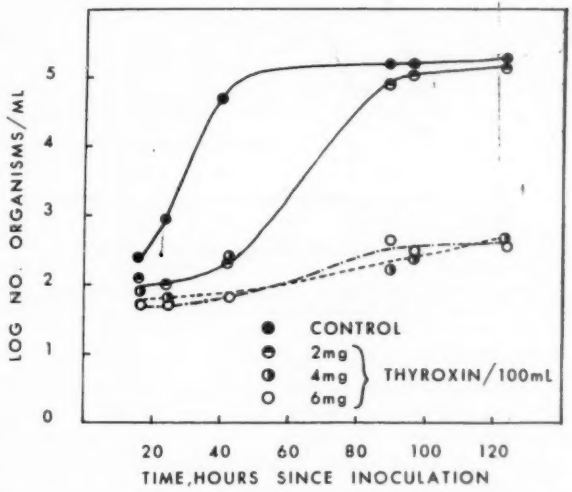

FIG. I. Effect of thyroxin upon multiplication of *Tetrahymena geleii* (Y) grown without aeration in Phelps' medium.

In figure II are presented the results as regards growth of a typical experiment in which the protozoa were grown with aeration on buffered medium with added calcium. At this lower concentration, the effect of thyroxin was similar to that in the earlier experiments, in that growth in the presence of thyroxin was characterized by a delayed recovery from a lag phase and a relatively slower rate of growth as compared to the control. The maximum population, however, approached closely, equaled, or even exceeded slightly (as it did in the experiment presented here) the maximum attained by the control culture.

Figure III shows the results as regards oxygen uptake for the experiment whose population data are shown in figure II.

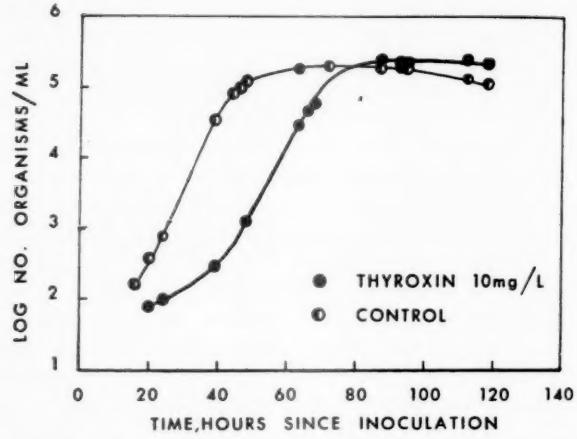

FIG. II. Effect of thyroxin upon multiplication of *Tetrahymena geleii* (Y) grown with aeration in Buffered Phelps' medium with added calcium.

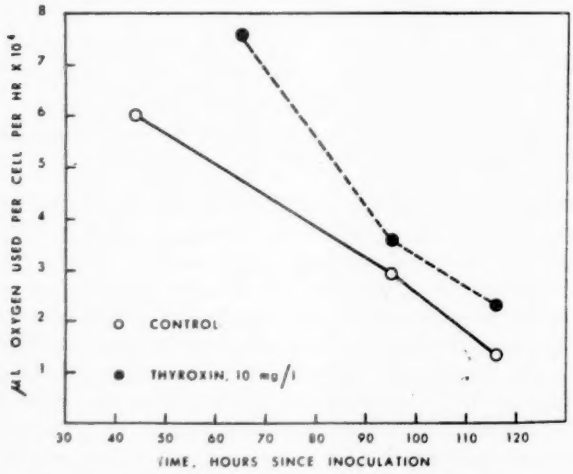

FIG. III. Effect of thyroxin upon oxygen consumption of the *Tetrahymena geleii* (Y) cultures whose growth is shown in (Fig. 2).

It is evident that the oxygen consumption of the cells grown in the presence of thyroxin was always greater than that of cells from the control culture. In this and other experiments of this type, the difference between oxygen consumption rates of the thyroxin and control cultures was considerably greater near the beginning of the cultures' growth than it was at the end. It was thought that this decrease in effect of thyroxin might be caused by destruction of the thyroxin; but experiments in which thyroxin-rich medium was added to cells whose oxygen uptake was being measured showed no effect of the thyroxin, either upon cells from control or thyroxin cultures, at least within 1 to 1.5 hours.

Discussion

The results of the present investigation clearly show that, at least in the concentrations used, thyroxin does not stimulate the reproduction of *Tetrahymena geleii* (Y), but instead is strongly depressing in action. The effect is similar in cultures grown with and without supplementary buffer to reduce pH changes caused by growth, and in cultures grown with or without aeration to maintain a constant oxygen tension. This must be reported simply as an observation, under relatively well-controlled conditions, which confirms earlier studies that showed depression of protozoan growth by thyroxin.

In the single concentration whose effect on oxygen consumption was tested, thyroxin was found to increase markedly the oxygen uptake of the protozoa. The increase in oxygen consumption was greater early in an experiment than it was towards the end. This may have been caused by destruction of the thyroxin, but the failure of thyroxin to produce an effect on oxygen consumption immediately after addition to a culture suggests that another factor (for example, the decrease with age of culture in the supply of a factor necessary for maximum respiratory rate) is responsible for the decline in oxygen consumption with time. Since the rate of oxygen consumption of the cells from the control culture likewise declines with time, it is probable that some such mechanism does come into play. That thyroxin should have no immediate effect on the oxygen consumption of the cells is not surprising, in view

of the findings of Davis *et al.* (1934 and 1936), that thyroxin increases the metabolism of the excised hearts of both the frog and *Limulus* only after these organs have been exposed to thyroxin for ten to fifteen hours, and of the usual observation in the clinical use of thyroxin or thyroid substance that the patient shows an increase in metabolic rate only at the end of a considerable time. It is interesting that, as regards time of onset of effect of thyroxin, such widely dissimilar organisms should resemble one another so closely.

It is tempting to speculate that the effect of thyroxin upon metabolism may be the cause of the depression which this substance exerts upon reproduction of *T. geleii.* If thyroxin imposes upon the cell an obligate spending of energy over and above the normal energy expenditure for normal physiological processes, and if the first stages of cell division constitute an endergonic process which can only occur if the cell has available a certain critical energy store, the postulated excessive rate of energy dissipation could hamper the accumulation by the cell of the energy necessary to effect division. If the effect of thyroxin upon the metabolism diminished with time, then one would expect the rate of multiplication of the cells exposed to thyroxin to approach more closely that of the cells of a control culture. The data presented here indicate that these circumstances both prevail, but considerably more data on the time relations of the effect of thyroxin upon the rate of oxygen uptake by the cells would be necessary to establish a good correlation between metabolic rates and relative rates of reproduction of thyroxin-treated and control cultures.

Summary

In concentrations of 10 mg. or more per liter, thyroxin hampers the multiplication of *Tetrahymena geleii* (Y).

The rate of oxygen uptake of *Tetrahymena geleii* (Y) is greater in cultures grown in the presence of thyroxin (10 mg./1) than in parallel control cultures. The difference in metabolic rate between the "thyroxin" organisms and the "control" organisms declines as the age of cultures increases.

The results presented are discussed.

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ASPECTS OF CANCER RESEARCH IN THE UNITED STATES OF AMERICA: A TRIBUTE TO GEORGE MILTON SMITH (1879–1951)*

STANHOPE BAYNE-JONES†

When a half-century has ended, or a new development has started, or a personage has died, human beings are accustomed to review the past, try to look into the future, and to memorialize the man. Around 1950 cancer research had its semicentennial reviews in many contexts. Scientific and material advances were described, and the lives of good men were recounted. None of these observances, however, could combine as well as this occasion does, a review of the past, the celebration of new developments illustrated by those taking place at the M. D. Anderson Hospital for Cancer Research, and the rendering of homage to the memory of a man—Dr. George Milton Smith—who contributed greatly to the achievements of the past fifty years and whose life and work will influence the next half-century, or longer. All of these elements are present in the composition of this occasion on which the Bertner Foundation Award has been presented posthumously to Dr. George Milton Smith, during the Sixth Symposium on Fundamental Cancer Research under the auspices of the M. D. Anderson Hospital for Cancer Research, on the eve of the realization of many of the hopes and ideals of its benefactors and planners.

Although I was associated with Dr. Smith for about twenty-five years, I cannot say I really knew him. Some who are here in this audience, and some who are in other places, knew him better than I did. But I wonder whether any one of us can claim intimate knowledge of the intricate combinations of practicality and mysticism, of sentiment and keen judgment, of generosity and shrewd estimates of costs that seemed to me

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to be innate in his great character. In the preparation of this lecture, I have had the advantage of talks with some of his family and friends, and I have had access to papers that few have seen. When I came to deal with his contributions to knowledge about cancer and his comments on cancer research, I realized more clearly than ever that one who is so far down in the abyss of administration as I am is not worthy of expressing scientific opinions. Nevertheless, admitting all of these shortcomings, I will do my best to speak frankly in honor of Dr. Smith and will venture to make some remarks about present trends in cancer research. Whatever this may be worth, I hope it will be accepted as an offering of thanks to you and to him.

Before I go on, I want to explain that in order to restrict the subject I have lengthened the short title that I telegraphed to Dr. Lee Clark last February. The title now is, "Aspects of Cancer Research in the United States of America." I feel sure that Dr. Smith would have approved of the more specific geographical designation. It is not only more correct, but also respectful of international relations to refer to U.S.A. rather than to U.S. when this country is the main subject of discourse. Dr. Smith was keenly aware of the internationalism and universality of science, and at the same time he appreciated the special qualities of the individual nations. He had friends and admirers in most of the countries of the world. While I am sure he would have approved of the use of the word "aspect" as a limitation upon the scope of any lecture on cancer, particularly one delivered by me, I imagine that he would have been intrigued by the astrological meaning of the word, signifying a glance conveying some influence, like the aspect of configurations of the planets, such as the zodiacal crab that was thought to have some influence upon the human beings upon which it looked. Certainly for the fate of men and women, there is such an aspect of work on cancer. I venture to suggest that this mystical element in "aspect" would not have been uncongenial to Dr. Smith, who had a feeling for the mysteries of the seas, from which he drew not only fish but also facts important for knowledge about cancer.

The life of Dr. George M. Smith is the main theme of this lecture, on which the other parts are variations. His interests were so broad and his activities so many, however, that I shall not try to tell you about all his long and useful life, but will be selective from the point of view of cancer research.

To an unusual degree, his life was co-ordinated. He had an integrated personality and consistent motivation. His earliest experiences were no doubt related to his lifelong interest in the sea and in fishes. His mature accomplishments in industrial medicine were related to his concern over environmental or occupational cancer. The civil and military service that he rendered to his country and to his state of Connecticut was of the same order and idealism as was his service to the investigation and control of cancer. His loyalties to organizations, like his loyalties to people, were generous and constant, whether in the field of cancer, or anatomy, or in any other endeavor. His abilities to organize and manage groups and to get things done found extensive and most beneficial expression in his work on cancer. When the same abilities were applied to problems of armored vehicles or the collection of rare books, they were equally effective. It will be difficult, therefore, to pick out from his life of 72 years the episodes that are most closely connected with his work on cancer. But this must be done for this lecture. Fortunately, I have had good advice from other friends to aid in the selection.

Of Danish parentage, George Milton Smith was born in Hong Kong, China, on July 5, 1879. His father was "a master of ships and merchant in the China trade." When he was nine years old, he went by sea from Hong Kong to Hamburg. There is evidence to suggest that his seafaring patrimony and this voyage across the oceans gave him an early sense of the vital importance of the sea and its creatures, which was later to result in some of his most valuable contributions to ichthyology and oncology. In addition, like a mariner whose charts were visions of undiscovered lands, he encouraged workers in areas of knowledge that had not appeared to be so close to cancer until he saw the connection.

At Yale, from which he graduated with the B.A. degree in 1901, he was, in his senior year, the organizer and captain of

the first Yale swimming team. What has this to do with cancer? This is mentioned not so much to show that he was strong of body and a good athlete, but as another variation on the marine motif, and as an example of his organizing ability and his capacity to secure a favorable opinion by casting a particular situation in a general mold. Apparently he had some difficulty in securing recognition of the sport. Finally, however, he persuaded Walter Camp to approve swimming as a "minor sport" at Yale. "As a final argument," Dr. Smith has written of this episode, "I told him about the mounting death rate in drowning and the desire of the state and national authorities to combat this by better teaching methods and the development of new swimming pools where the public would learn more about the subject. This argument," Dr. Smith concludes, "seemed to have great weight with Mr. Camp." Thus at the age of 22, the young promoter secured the satisfaction of a personal desire which was also something for the good of all. In addition, all the elements and objectives of later campaigns against cancer are in this simple account of his argument with Walter Camp. There is the emphasis upon a fear-motive and a plan for the development, by training, education, and new facilities, of ways and means for preventing some accidental deaths and saving lives. It is a small happening in a life of large achievements, but it is an example of his pattern of persuading the powers-that-be to do something.

A manuscript written by him about fifty years later contains another remarkable example of Dr. Smith's early serious use of an opportunity for learning outside of his college courses and of his interest in cancer. In 1950, he started to prepare a history of cancer research in honor of the 20th Anniversary of the Anna Fuller Fund. The book is an immense volume of heavy paper leaves between which are inserted loosely sheets of notes and drafts of sections typed on yellow paper; many annotated in his handwriting. The volume was to be entitled "Cancer Crusaders" or "Crusaders of Cancer Research." This choice of emotional words for a romantic title of what must be a grim history was very disclosive to me, and moving. It shows, as do many other remarks in the manuscript of this history, how intensely he felt. The history is valuable for what

it tells about George Smith, as well as about Johannes Mueller. Like his pencilled notes of his own appreciation on the covers of his personal collection of reprints of his scientific papers, it is an autobiographical document. I am privileged to have seen both of these sources.

In his "Cancer Crusaders," the earliest reference to an American event that influenced Dr. Smith is his note about the lectures "concerning the causation of carcinogenic and other growths," which Dr. J. G. Adami delivered at Yale in December, 1900. At this time, George Smith was still an undergraduate, in his senior year. He credits Adami with "altering many of us to cancer." When he came of age, he was already aware of the cancer problem.

After a successful four years at the College of Physicians and Surgeons of Columbia University, he received the M.D. degree in 1905. Of the next five years spent in training in surgery at Presbyterian Hospital and other hospitals in New York, it seems to me that the last year, 1909–1910, when he worked in surgical pathology under Dr. Eugene L. Opie, was the most important for its bearing on his future work in cancer.

In 1910, he went with Dr. Opie to work in the Department of Pathology at Washington University School of Medicine in St. Louis. Here he rose to the position of Associate Professor of Pathology and in 1915 became Director of the Barnard Skin and Cancer Hospital. He was in contact with Dr. Leo Loeb and from him received inspiration and broadened biological outlook on cancer. About six years of his life, from 1910 to 1916, were spent in this environment of experimental and clinical work on cancer.

During this period he published his first papers on cancer research. These reported the results of his attempts to produce cancer in animals by transplanting organs, chiefly gall bladder and portions of the stomach, to foreign sites in the bodies of the same animals where they would be subjected to changes in chemical environment and possibly other growth-influencing factors. He did not produce cancers by these methods, but he learned something about the changes in cells growing in new locations, and his own ideas and enthusiasms grew because of the influence of Dr. Opie and Dr. Carrell, to whom he gives

credit for arousing his interest in transplantation of organs. Perhaps his deep concern for gastric cancer began at this time when he was seeing the problems experimentally, in sufferers in the hospital, in operations, and at the autopsy table. It is not stretching probabilities too far to assume that in this period he formed the purposes of his later years of support and encouragement of workers on gastric cancer and the important Gastric Cancer Conferences which he arranged and conducted for the National Cancer Institute thirty years and more later, from 1943 to 1950.

Today we do not hear much talk about cancer arising from misplaced embryonic rests, or from tissues transferred to foreign sites. We do, however, hear a great deal of discussion about the cause and control of cancer through chemical action, which was an underlying thought in his early transplantation experiments. These are the subjects of a vast amount of current investigation, including studies of carcinogens, metabolites, genes, and viruses in relation to the growth and behavior of cells. I recall many times since 1937 particularly, when Dr. Smith said that the cancer problem would be "licked" by chemistry; and I understood him to mean both the biochemistry of metabolism and chemotherapy.

In 1916, Dr. Smith left St. Louis and went into private surgical practice in Waterbury, Connecticut. In time he became the medical director of the Scoville Manufacturing Company and the American Brass Company. This marked the beginning of his interest and work in industrial medicine. It led to his great service in civilian defense in the state and nation in two world wars. Not even his active duty in France as a Captain in the Medical Corps of the Army with the Yale Unit, Mobile Hospital No. 39, in 1917–1919, impeded his advance in the field of industrial medicine.

I knew little about his life from 1916 to 1932. He might have been diverted to fashionable private practice. I have wondered what kept him on his zodiacal course. I assume that the explanations are to be found in his ideals and in his relative economic security. Fortunately, when he returned to Connecticut, he came back among sympathetic friends at the Yale University School of Medicine. They encouraged his interests

in research and in developing co-operative work on cancer. The going was hard, but the spirit was there.

In 1932, at the end of these sixteen years, Dr. Smith stands out clearly as an original investigator of cancer and as an organizer and director of new agencies for the encouragement and support of work on cancer. Among these new agencies, The Anna Fuller Fund was the first and most important in his life. He was adviser and medical director of this Fund from 1932 until his death in 1951. Through his influence, The Anna Fuller Fund became a catalyst, an innovator, a supporter, and a solace for investigators of cancer throughout the world.

After 1916, among the friends around him at his home at Pine Orchard by the Sea, on the Connecticut shore, was Mr. Egbert Chaplain Fuller, whose wife had died of cancer. I have not seen any records of Dr. Smith's talks with Mr. Fuller. But I like to imagine their conversations, seriously and hopefully discussing the need to support cancer research—the doctor suggesting ideas and the industrialist weighing his means.

This was in the period of "the great darkness" for cancer. The American Cancer Society, founded in 1913, was almost the only public organization with the courage and optimism to promote work on cancer during these fourteen years after World War I. Memorial Hospital, Barnard Hospital, Lankenau Hospital, and Crocker Institute were about the only specialized cancer institutes. Only a few prominent men, notably Dr. Ewing, believed that something could be done by basic work on cancer. I know, however, from personal talks with Dr. Ewing some years later, in 1937, when I consulted him about a program for The Jane Coffin Childs Memorial Fund for Medical Research, he was skeptical. He said that anyone going after the formal genesis of cancer—the nature of the malignant process—would be getting out on a limb. Many investigators felt that to enter cancer research was to go into a blind alley. They knew that work on problems of cancer wouldb reak a man's heart, if not his reputation. Perhaps the total fund for cancer research in 1930 amounted to $100,000 a year. Relative poverty and despair were the lot of projects and investigators.

Not so was Dr. George Smith. In 1931, he began to urge the Dean of the Yale Medical School to ask the members of the

faculty what they might like to do in cancer research. At first there were no answers. This was interesting but not discouraging to Dr. Smith, who was, no doubt, continuing his talks with Mr. Fuller. Mr. Fuller died on March 5, 1931, and in November, 1932, The Anna Fuller Fund, for which he had provided in his will, was established.

Dr. Smith was the Fund's first "adviser" and "medical director." The first minutes of the Board of Trustees of The Anna Fuller Fund are eloquent in their unadorned confidence in their adviser. In "Cancer Crusaders" Dr. Smith refers to his appointment and to his advice to the Trustees "not to be too cautious," because, as he expressed it, "cancer crusaders must be captains courageous."

The year 1932 was the beginning of the new era in the support of work on cancer and in cancer research in the United States of America. Twenty years of ever increasing gains in funds, facilities and investigators began that year. The International Cancer Research Foundation was established by Mr. William H. Donner on June 8, 1932. The Anna Fuller Fund was officially established on November 10, 1932. Dr. Smith, at Yale, made the first moves to establish "The Atypical Growth Study Unit," which began to hold its meetings the next year. In 1932, Dr. Smith first began to work on tumors in fishes at the New York Aquarium, in association with Dr. Myron Gordon, beginning activities that led to discoveries, to initiation of a new field of comparative oncology, and to the famous Conferences on the Biology of Normal and Atypical Pigment Cell Growth—the so-called Melanoma Conferences—in 1946 and 1949.

Just before 1932, the American Cancer Society, which had been founded in 1900, was strengthened and increased its activities in a direction that led to its present phenomenal growth and success, following its reorganization in 1945, in raising many millions of dollars a year for education, service, and research.

In 1929, Dr. C. C. Little established the Roscoe B. Jackson Memorial Laboratory at Bar Harbor, Maine. This Laboratory has had a notable career of research and service in genetics and has supplied the world with indispensable pure-line strains of mice and discoveries from original research.

In 1937, the National Cancer Act was passed by Congress. Although I was a member of a committee which Dr. Parran appointed to give suggestions and advice on a program for the National Cancer Institute, I had no conception, when we published our statement in 1938, of the enormous part that the U.S. Public Health Service would play in the conduct of original investigations of cancer by its own personnel and in the support, amounting to millions of dollars a year, of research, training, and teaching of cancer in medical schools and hospitals throughout the country. The ideas in our committee report of 1938 still seem to me to be almost as valid today as they were then. But we had not, I must admit, estimated the costs. Dr. Smith had great influence upon the development of the program of the National Cancer Institute.

In 1937, The Jane Coffin Childs Memorial Fund for Medical Research was established, with headquarters at Yale University, to provide support and encouragement for study primarily of the causes and origins of cancer and of the treatment of cancer. The Fund came into being at a critical time. The results of its work have been both tangible in results and intangible in what it has done to foster ideas and provide opportunities for men to work on cancer. Its early records read like the records of the Committee on Growth ten or more years later, as far as general concepts are concerned. It, as well as other independent research foundations, continues to have special functions to perform, and may well anticipate results that are not measurable in the dollar values of its grants. Dr. Smith was a member of the Board of Scientific Advisers of this Fund. In his letter of acceptance of this appointment in May, 1938, he wrote modestly: "The appointment comes to me as a great honor and as a longed-for opportunity to assist in an undertaking generally believed to represent the most important effort ever made to explain the cancer question."

As Dr. Smith's concern with cancer was almost as broad and deep as the whole subject, there are oceans of records to be explored by any conscientious historian of his life and work during the period from 1932 until his death on February 26, 1951. Even on a limited voyage, I collected a large cargo of notes, and have too much material to be spread out in this lecture. It is

pertinent to discuss Dr. Smith's collaborative research during this period.

In 1935 and 1936, Dr. Smith, in association with Drs. Edgar Allen, William U. Gardner, and Leonell Strong published several papers on the induction of mammary cancer in male and female mice by large injections of estrogens. His personal comments written in pencil on the covers of reprints of these papers are interesting. On the cover of the first paper on the stimulation of mammary growth by large amounts of estrogenic hormone he wrote: "Following visit to London and Paris." It was on this visit that he saw Kennaway and Lacassagne. Through the latter he bought a new estrogenic compound (benzogynestryl) first used by Lacassagne as "folliculin benzoate." On one paper, in 1936, in this connection he wrote: "The Yale team.—First American paper on the experimental production of cancer in the male mouse following injection of female sex hormone (chemicals brought from Paris)." On the cover of another paper in 1936 he wrote: "First report of sarcomas following female sex hormone injection into mice." In 1940, on the cover of a paper by Burr, Smith, and Strong, he noted: "Pioneer studies on electrical potentials in cancer."

In the years following 1936, he published alone, or with such collaborators as Drs. Myron Gordon and Nigrelli, a number of papers on tumors in fishes, electric eels, and turtles, and, in 1937, an original account of lymphocystis disease in *Angelicthys*, which, as he noted on the cover, was "the first description of this remarkable disease along the Atlantic seaboard." He was fascinated by Lucké's work on virus nephromas of frogs and on Gordon's genetic-carcinogenic investigations of pigmented tumors in fish. He was happy in this research on malignancies in aquatilia.

He was appreciated at the New York Aquarium, as he was in other institutions. Dr. Fairfield Osborn, President of the New York Zoological Society, in a recent letter to Dr. Lee Clark, has so well expressed the sentiments of many men that this eulogy is made eloquent by quotation from him. "It was through his efforts," wrote Dr. Osborn, "that an extensive program for the investigation of abnormal growth in fishes was instigated, leading eventually to the establishment of the Genetics Laboratory

(at the Aquarium) and to the present position we enjoy in the field of comparative oncology."—"It was Dr. Smith's firm conviction that the cancer problem must be attacked on the broadest possible front. Vital facts were to be learned from tumors in fishes, turtles and alligators—that is where the Aquarium fitted into the over-all program of cancer research."—"He was, however, a man above and beyond any stated record. His extraordinary gift of encouraging others, his invigorating and delightful personality, and his belief in the values of mutual cooperation between all men dedicated to science, seem to me unique."

In his will Dr. Smith bequeathed to the Historical Library of the Yale Medical Library his collections of reprints and books. Among these was "one of the finest private collections of early European scientific works on fishes." Professor Daniel Merriman, describing these books, wrote: "As a private collection of the ichthyologies of the sixteenth and seventeenth centuries, Dr. Smith's library is probably unsurpassed."—"No account of George Milton Smith or his library would be complete without reference to his devotion to the early and sometimes mystic beginnings of science." References are then made to early Roman poems about fishes, and "Finally, perhaps the book which meant most to Dr. Smith was his beloved Hildegarde (1533), whose *Physica*, was originally written in the first half of the twelfth century." Of the Holy Hildegarde, Abbess of Bingen on the Rhine, Dr. Smith himself wrote that she was "an extraordinary genius,—a mixture of ecstatic visions, sacred music and mystical mediaeval science.—Her book on fishes—is said to be of some faunistic importance, as it describes over thirty fishes to be found in that part of the Rhine and its tributaries."

In preceding sections of this review four stages of development of support of research, education, and training in cancer have been indicated in relation to the half-century, from 1901 to 1951, of Dr. George Smith's life as a "cancer crusader." These were (1) the period of "darkness" and poor support (1901 to 1931), (2) the period of the establishment of new foundations and agencies and the beginning of their increasing moral and financial support (1932 to 1937), (3) the acceleration of active support by the American Cancer Society and the National Cancer Institute (1938 to 1945), and (4) the current period (1946 to

1952) of tremendous increase in availability of funds, facilities, and personnel as the result of the change in attitude toward cancer, as illustrated by the reorganization of the American Cancer Society in 1945 and the greatly increased federal appropriations for the National Cancer Institute and program of the U.S. Public Health Service starting in 1948. Enough has been said previously about the first three periods. A few spectacular facts and figures from the current period will be presented here. They will show how even a guarded optimism toward solution of the cancer problem has been so inspiring that great public organizations have been assembled and vast new facilities have been produced, such as the Sloan-Kettering Institute for Cancer Research, the Lankenau Hospital Research Institute and the Institute for Cancer Research, the M. D. Anderson Hospital for Cancer Research, and other similar powerful institutions. The entry of the Atomic Energy Commission into the field of work on cancer symbolizes the chain reaction that has been going on in support of cancer since the end of the war. In this lecture I cannot even enumerate the main details of this vast and seething activity. Fortunately, I do not need to try to do so, because all who are interested now have available to them the brilliant and comprehensive report by Stella L. Deignan and Esther Miller, entitled "The Suport of Research in Medical and Allied Fields for the Period 1946 through 1951" (*Science*, 115, 321, March 28, 1952). This report is a publication from the Medical Sciences Information Exchange Division, Division of Medical Sciences of the National Research Council. It is indeed, as the Policy Committee believes, an indication of the success of co-operative effort in pooling information about grants and expenditures for research, about the subjects of research, and about the investigators and the institutions to which they are attached.

The report is an analysis of 12,923 grants from 1946 to 1951, based on research grants and contracts awarded by the government and by the larger private foundations. The number of undertakings and the amount of funds available would have been larger if it had been possible to include the support given by universities, schools, and hospitals directly to research in medical and allied fields. But even without this portion, the

figures are very large. A few of the data relative to cancer research are extraordinarily interesting.

In 1946, the total amount for cancer was slightly under $1,000,000 for the year. This increased each year, and in 1951, the funds available for cancer research were nearly $7,000,000 in that year. When one compares this with a time, about twenty years ago, when an annual amount of $100,000 a year for cancer research was thought to be large, one can appreciate what a surge upward has been made by support for cancer research since the days when one chief of a laboratory used to play the piano at concerts to raise a few dollars for some chemical investigations of malignant and normal growth. Of the total of approximately $135,000,000 granted for medical and related research during this period of six years, nearly $28,000,000 has been devoted to cancer research. To the understanding hearer and reader of these statements, the money-figures assume the forms of workers and their laboratories, of organizations and their activities, and the innumerable members of the human race who are so fatefully concerned with the solution of the problem of cancer. I feel that I do not need to say more to emphasize the meaning of this support. Whether or not it is enough to secure the desired results, no one can say with certainty, because no one with certainty can ask exactly the specific questions that need to be answered. Large as it is, the amount is pitifully small compared with sums spent on industrial research, or on research on destructive weapons. In any case, considering the urgency of the need for solution of the cancer problem, the amounts and facilities for research should be continuously increased, provided, of course, there are men and women of skill, ideas, and energy ready to use the means for the attack. Of such persons more and more are being bred and trained, since the managers of these funds know that ideas cannot be created by money and hence are using some of the money to bring on more people who alone can provide the ideas.

The power given to research by such great financial resources is obvious, and the advantages of concentration of control of large portions of these resources are well understood. There are, however, some dangers in the system. It is not out of place to mention them here. Indeed they are recognized by the Na-

tional Cancer Institute, the American Cancer Society, the Committee on Growth, and the boards of foundations. The system of grants-in-aid under concentrated controls tends to foster project research that shows promise of results. The long-range, basic scientific work and training is not apt to receive support in competition with the more specific short-term projects. The system tends to produce directors, supervisors, and co-ordinators with great powers over investigative undertakings. There is a danger that interest may be diverted into fields in which the financial rewards are attractive while the ground is not worth cultivating. There is a danger that the field in which some essential truth may be found will be by-passed because the means for entering it are lacking as the result of disapproval of a request for funds. Dr. Smith understood all of this very well and was able, through The Anna Fuller Fund, to encourage "pilot" studies. But far more unrestricted support of basic research and training is needed in the medical and allied fields, especially in these critical times.

It is my opinion—stated without any claim for originality since MacCallum, Foulds, Kidd, and others have expressed it much better—that there is something in the processes or characteristics of the malignant cell which will not be disclosed by investigations of normal growth. There should be more direct study of cancer, and, whenever possible, cancer in human beings. I have had the experience of participating in making grants for investigation of the biochemistry of cancerous tissue, for example, only to find that after a contact with the disheartening difficulties, the investigator has found it more convenient to transfer the studies to normal tissues. I can recall, for example, transamination studies on experimental hepatomas transferred to slices of normal livers, which could be obtained in abundance and with ease. I could add other examples. My hope, however, is to urge more frontal attack on cancer and the use of malignant tissues for the fundamental studies of the processes of malignancy. Although oriented to the investigation of the abnormal, such studies are certain to elucidate the normal. The knowledge of the functions of the thyroid, pituitary, adrenals, and pancreas did not come from infinitely prolonged studies of the normal glands, but came relatively quickly from the studies

on goiter, acromegaly, Addison's disease, and diabetes. The same may be said of the knowledge of renal function that has been gained from experiments performed originally by nature upon the kidneys. There is a better prospect of knowing about the normal by investigation of the pathological than there is of knowing about the abnormal by investigation of the normal. I am convinced that this applies to cancerous growth.

Having ventured to express my personal opinion that the central problems of cancer are the special metabolic, chemical, and biological processes of malignant growth, I wish I knew at this moment what Dr. Smith would think about this. As he kept his own research interests focussed on cancerous tissue, I imagine that he would agree in general. In his wisdom and breadth of sympathy, however, he would not have excluded work on any problem, the solution of which might be expected to give more knowledge about cancer.

Official tributes to his wisdom, sympathies, and accomplishments came to him with increasing frequency toward the end of his life. Unfortunately, two honors that he would have enjoyed most deeply were awarded after his death. The first was the Cross of Knighthood of Danneborg, conferred upon Dr. Smith by the King of Denmark on May 15, 1951. The second was the Bertner Foundation Award, presented posthumously in his memory tonight. He would have been proud and happy to receive this award that bears the name of his friend who gave it. He would have valued the award for all of its deep meanings, as he would have cherished the honor and pleasures of this gathering of zealous friends devoted to the common cause of overcoming cancer.

CALLING ATTENTION TO:

Items of Possible Interest to Our Friends

June, 1952

Clinical: PM Aggeler & Keene Co. describe plasma thromboplastin deficiency disease (*Proc Soc Exper Biol Med* 79/692/52). J Eschler shows slight continuous pressure strain on teeth is sufficient to cause necrobiotic change in peridontal membranes (*Ztschr Micro-Anat Forsch* 58/100/52). T Fog finds ACTH helpful in disseminated sclerosis (*Acta Endocrinol* 9/213/52). WD Forbus offers 2nd vol of famed *Reaction to Injury* (Wms & Wilkins, Balt., 52, Vol 1, 816 pp, $9; Vol 2, 1132 pp, $20). SI Griboff reviews steriods in cancer (*Arch Int Med* 89/685, 812/52). T McKeown & JR Gibson estimate period of human gestation at 280 to 358 days (*Brit Med J* 1/938/May 3, 52). J McMichael is hopeful about management of hypertension (*ibid*, p 933). KG Ober discusses cyclic body temperature changes in relation to ovarian function (*Klin Wchnschr* 30/357/Apr 15/52). Our EJ Poth reviews intestinal antisepsis in surgery (*West J Surg Obs Gyn* 60/205/52). RB Rees & Co recommend 70% sulfadiazine & 30% urea powder for local application in impetigo (*Calif Med* 76/344/52). RW Rundles & WB Barton confirm DA Karnofsky & Co (*Arch Int Med* 87/477/51) that triethylene melamine orally is useful in neoplastic disease (*Blood* 7/483/52). DJ Sandweiss, MH Sugarman & LR Dragstedt sympose on peptic ulcer (*Rev Gastroenterol* 19, 271–286/52). A Schaede describes cardiac tricuspid atresia (*Deutsch Arch klin Med* (199/102/121/52). G Schneider & C Wunderly propose rapid paper electrophoresis method for clinical lab use (*Schw Med Wohnschr* 82/445/Apr 19/52). GA Schumacher edits symposium on multiple sclerosis & demyelinating diseases (*Am J Med* 12/499–595/52). NB Talbot & Co offer *Functional Endocrinology from Birth Through Adolescence* (Harvard Univ Press, Cambridge, Mass, 52, 672pp, $10). F Venulet & Z Moskwa show tobacco smoking reduces ascorbic acid levels (*Pol Tyg Lek* 7/281/52). B Zimmermann & OH Wangensteen discuss water intoxication in surgical patients (*Surg* 31/654/52).

General: FT Gardner finds Robert the Bruce (1274–1329) no leper (*Surg Gyn Obs* 94/373/52). KD Keele well describes *Leonardo do Vinci (1452–1519) on Movement of the Heart and Blood* (Lippincott, Phila., 52, 160pp, $10), with foreword by C Singer. For a short general note on Leonardo, see article on his work as artist and scientist (*Texas J Sci* 4/1/52). HW Smith is critical and autobiographical about *Man and His Gods* (Little Brown, Boston, 52, 511pp, $5), with note by A Einstein. RW Sperry analyses neurology & the mind-brain problem (*Am Scient* 40/291/52). Festschrift to E Warburg includes fine papers on medicine and history with note by N Bohr on medical research (*Acta Med Scand*, Suppl 266, 52, 972pp).

PRECLINICAL: E Boyland reviews biological effects of radiations & radiomimetic drugs (*Endeavor* 11/87/52). *Un Colloque tenu a Mècanisme de la Narcose* contains stimulating ideas (Stechert, Paris, 51, 215pp, $5.75). J Del Castilo & L Stark indicate that acetylcholine released at motor endplate is dependent on calcium ion concentration (*J Physiologie* 116/507/52). E DeRitter & Co show 65% hydrazine derivatives of isonicotinic acid (for Tb) excreted in urine in 48 hrs. (*Proc Soc Exper Biol Med* 79/654/52. H Fischer well reviews histamin & antihistaminics (*Schw Med Wohnschr* 82/465, 504/Apr 26, May 3/52). GC Gotzias & VP Dole show high histaminase in mitachondria of liver cells & describe method of estimation (*J Biol Chem* 196/235/52). P Marquardt & C Vogg report cholinergic factor in honey (*Arznei Forsch* 2/152/52). G Martin & JM Beiler show oral hyaluronidase inhibitor, a phosphorylated hesperidin, inhibits fertility in female (*Science* 115/402/Apr 11/52). SO Nielsen & T Rosenberg propose mechanism of gastric acid secretion by phosphate bond energy (*Compt rend Lab Carlsberg* 27/437/51). G Rajka discusses hypersensitivity to para type organic compounds (*Acta Allergol* 5/11/52). WT Salter offers well documented conventionally organized *Textbook of Pharmacology: Principles and Application of Pharmacology to the Practice of Medicine* (Saunders, Phila., 52, 1240pp, $15). FW Schueler correlates muscarinic activity with polarizing forces in molecule (*Science* 115/548/May 16/52). M Solotorovsky & Co find pyrazinoid acid amide orally active against TB (*Proc Soc Exper Biol Med* 79/563/52). M Wertheimer & WD Ward confirm JD Hardy & Co (*Science* 114/149/51) that skin temperature influences pain threshold to thermal radiation (*Science* 115/499/May 2/52).

HOT JULY, 1952

HOT & DRY READING: W Chambers' *Witness* is an interesting document for current psychiatry (Random House, NY, 52, 808pp. $5), & is well reviewed by S. Hook (*NY Times Book Rev*, May 25/52). G Horrax reflects on *Neurosurgery: An Historical Sketch* (CC Thomas, Springfield, Ill, 52, 135pp, $2.5). PM Dale examines the great post-mortemly in *Medical Biographies* (Univ. of Oklahoma Press, Norman, 52, 220pp, $4). RM Green offers *A Translation of Galen's Hygiene*, with introduction by HE Sigerist & translation by S Licht of D LeClerc's of Galen (*Historie de la Medicin*, Amsterdam, 1723) (CC Thomas, Springfield, Ill, 52, 297pp, $5.75). A Loos dramatizes Colette's *Gigi* (Random House, NY, 169pp, $2.5). MJ Moroney tells to derive *Facts from Figures* (Pelican Book, Baltimore, 52, 472pp, $1.25). EH Mudd well advises on *The Practice of Marriage Counseling* (Asso. Press, NY, 52, 336pp, $4.5). M Pinner & BF Miller find good stories *When Doctors Are Patients* (Norton, NY, 52, 264pp, $4). G Sarton well devises *A Guide to the History of Science* (Chronica Botanica, Waltham, Mass., 52, 3200pp, $5). EWF Tomlin epitomizes the works of *The Great Philosophers: The Western World* (AA Wynn, NY, 52, 296pp, $3). R Truax pleasantly biographizes *The Doctors Jacobi* (Little Brown, Boston, 52, 270pp, $3.5).

DRY BASE: M Ashford edits symposium on musculoskeletal system (Macmillan, NY, 52, 380pp, $6.5). C Barigozzi in studying isolated chromosomes and spiralization cycle shows on general model of the resting nucleus is yet available (*Experient* 8/133/May 15/52). E Braun-Menedez proposes renotrophin as protein fraction regulating renal growth & function, with hypertension resulting from excess (*Acta Physiol Latino-Amer* 21/1/52). LJ Cole & Co protect mice against x-irradiation by spleen hemogenates (*Proc Soc Exp Biol Med* 80/112/52). A Frey-wyssling edits *Deformation & Flow in Biological Systems* (Interscience, NY, 52, 560pp, $11.50). WH Gaylord Jr & Co find vaccinia virus growing at nuclear membranes (*Proc Soc Exp Biol Med* 80/24/52). SP Hicks & PO Montgomery find subependymal cells in brain are radiation sensitive (*ibid* p15). O Krayer reviews antiaccelerator cardiac agents (*J Mt Sinai Hosp* 19/53/52). J Lodder & NWJ Kreger-van Rij offer a taxonomic study of *The Yeasts* (Interscience, NY, 52, 690pp, $19.8). EW Maynert & Co smypose on fate & mode of action of barbiturates (*Fed Proc* 11/625/52). WD McElroy gives evidence for intermediates in mutation (*Science* 115/623/June 6/52). M Monnier & J Babel show that retinal fusion frequencies decrease peripherally as cones diminish & that objective fusion frequency is lower than subjective (*Helv Physiol Pharmaco Acta* 10/42/52). EP Pick has fine 80th birthday volume (*J Mt Sinai Hosp* 19/1/358/52). MS Raben & VW Westermeyer separate diabetogenic factor of anterior pituitary from growth hormone (*Proc Soc Exp Biol Med* 80/83/52). MW Robinson & WL Voegtlin deny antigenicity of alcohol (*Quart J Studies Alc* 13/196/52). S Sangvichen finds CO_2 can reverse direction of embryonic chick heart beat (*Anat Rec* 112/529/52). A Stoll & E Wedemann well review chemical relations of chlorophyll (*Fortschr Chem Forsch* 2/538/52). EM Vicari & Co show glucose, epinephrin & sex steriods reduce convulsive seizures (*Proc Soc Exp Biol Med* 80/47/52).

HOT CLINICAL: IM Allen discusses forced thinking as part of epileptic attacks (*New Zealand Med J* 51/86/52). HH Anderson & Co discuss amebiasis in Arab east (*J Roy Egypt Med Asso* 35/225/52). WG Bigelow & Co describe radio frequency rewarming in resuscitation from hypothermia (*Canad J Med Sci* 30/185/52). KW Cross & TE Oppe find infants have more active chemoreceptor respiratory reflexes than adults, but this is poor in anoxia (*J Physiol* 117/38/52). GG Hartman edits symposium on world population & birth control (*Ann NY Acad Sci* 54/731–867/52). PH Knapp considers amphetamine addiction (*J Nerv Ment Dis* 115/406/52). H Mautner reviews transfer of drugs into the milk (*J Mt Sinai Hosp* 19/83/52). Our ON Miller & RH Rigdon show pancreatic fibrosis from undernutrition (*Proc Soc Exp Biol Med* 80/165/52). CE Palmer hopefully revalues BCG vaccination (*Lancet* 1/935/May 10/52). HE Rosvold & Co find electroshock hypertrophies adrenals, with effect prevented by anesthesia (*Proc Soc Exp Biol Med* 80/60/52). H Selye tells *The Story of the Adaptation Syndrome* (Acta, Montreal, 52, 225pp $5). ER Trethewie

finds p-amino salicylate inhibits histamine release (*Med J Austral* 1/638/May 10/52).

AUGUST, 1952

CLINICAL: J Barrow & JL Tullis show progressive less sensitivity to total x-radiation from lymphocytes through erythropoietic & granulocytic cells to intestinal epithelium & spermatozoa, without injury to reticulo-endothelial cells (*Arch Path* 53/391/52). JC Bourne & Co recommend succinoylcholine as short safe muscle relaxant in surgery (*Lancet* 1/1225/June 21/52). JF Brailsford discusses osteogenesis exhausta (*Med Illust* 6/167/52). HR Brown Jr & Co describe *Clinical Ballistocardiography* (Macmillan, NY, 52, 188 pp, $5.5). LI Dublin & HH Marks analyse mortality among insured overweights (*Proc Asso Life Ins Med Dir Am*, 52). PS Hetzel notes serum globulin depression during dextran use (*Med J Austral* 1/657/May 17/52). N Jolliffe, FF Tisdall & PR Cannon give *Clinical Nutrition* (Hoeber, NY, 52, 937pp, $12). R Lecog & Y Dhotel show hypotensive action of axerophtol, a synthetic vitamin A (*Compt Rend Soc Biol* 145/1820/51). P Mullahy edits *The Contributions of Harry Stack Sullivan* (1893–1950), great Baltimore psychiatrist—"A Symposium on Interpersonal Theory in Psychiatry & Social Science" (Heritage, NY, 52, 238pp, $3.5). R Platt discusses adaptations in renal failure (*Brit Med J* 1/1313/June 21/52). M Rosecan & Co describe clinical syndrome of orthostatic hypotension, anhidrosis & impotence (*Circul* 6/30/52). WG Schenk & Co conclude that moderate hemolysis in blood stored for human transfusion is no contraindication to its use (*Surg* 31/870/52). RC Scott & Co find papaverine derivative, dioxyline phosphate, safe & helpful in angina pectoris (*Circul* 6/125/52). TH Spaet proposes relation between blood coagulation system & blood vessel walls, with vascular lesions involved in etiology of purpura (*Blood* 7/641/52). HB Stover & Co analyse carbohydrate metabolism in shock (*Brit J Exp Path* 33/131/52). WF Von Oettnigen offers *Poisoning: A Guide for the Clinician* (Hoeber, NY, 52, 534pp, $10). K Weinbren describes pathology of hepatitis, showing bile thrombi & necrotic liver cells (*J Path Bact* 64/395/52). T Winsor & Co find penta-erythritol tetranitrate safe & useful in cardiac conditions (*Angiol* 3/1, 16, 20/52).

PRECLINICAL: H Aebi shows tissue swelling related to potassium content (*Helv Physiol Pharmacol Acta* 10/184/52). WJ Beecher calls attention to the unexplained direction sense of vertebrates (*Sci Month* 75/19/52). WD Celmer & HE Carter describe the chemistry of phosphatids & cerebrosides (*Physiol Rev* 32/167/52). L Dautrebande discusses physiological & pharmacological characteristics of liquid aerosols (*ibid* p 214). H Downey studies megaloblast & normoblast formation in treated anemias (*J Lab Clin Med* 39/837/52). LC Dunn edits *Genetics in the 20th Century* (Macmillan, 52, 634pp, $5). V DuVigneaud pleasantly relates the evolution of his studies on the sulfur of insulin to biologically labile methyl groups, transmethylation, posterior pituitary hormones, & the significance of single

carbon fragments in metabolism, in "A Trail of Research in Sulfur Chemistry and Metabolism" (Cornell Univ Press, 52, 203pp, $3). T Grima implies that digitalis improves cardiac contractility by restoring adenosine triphosphate (*J Pharmacol* 105/178/52). WR Hess surveys the implications of findings in experimental physiology on current concepts in psychology (*Helv Physiol Pharmacol Acta* 10/85/52). I Mandl & Co show solubilizing power of soluble salts of adenosine triphosphate (*Biochim Biophys Acta* 8/654/52). HG Petering discusses folic acid antagonists (*Physiol Rev* 32/197/52). EA Pinson surveys water exchanges & barriers (*ibid* p 123). JT Randall & Co reveal aspects of collagen structure (*Nature* 169/1029/June 21/52). Note *Symposium on Pituitary-Adrenal Function* (AAAS, Washington, 52, 224pp, $4). LG Welt & WP Nelson show no mechanism responsible for water excretion other than elimination of antidiuretic hormone responds to hypotonicity of body fluids (*J Applied Physiol* 4/709/52).

SEPTEMBER, 1952

THE CLINIC: AL Bloomfield offers helpful bibliography of scarlet fever (*Stan Med Bull* 10/114/52). CW Clarke wisely condemns medical examination & certification of prostitutes, in support of AMA policy (*PG Med* 12/162/52). AH Douthwaite & AB Shaw recommend sucking milk-alkali tablets to reduce gastric acidity (*Brit Med J* 2/180/July 26/52). JP Greenhill well reviews *Analgesia & Anesthesia in Obstetrics* (CC Thomas, Springfield, Ill, 52, 91pp, $2). M Prinzmetal & Co analyse *Accelerated Conduction* (Grune & Stratton, NY, 52, 118pp, $4). T Winsor & Co find pentaerythritol tetranitrate long acting in coronary insufficiency (*Angiol* 3/1/52).

THE LAB: BC Abbott & Co define negative work (muscle lengthening) & note it takes 1/4 as much O_2 as positive work in muscle shortening (*J Physiol* 117/380/52). A Albert surveys ionization, pH & biological activity (*Pharmacol Rev* 4/136/52). ESG Barron edits *Modern Trends in Physiology & Biochemistry* (Acad Press, NY, 52, 560pp, $8.5). HK Beecher pontifiacts on measuring subjective response in experimental pharmacology (*Science* 116/157/Aug 15/52). R Berfenstam finds plasma zinc level 110 microgr %, while low blood corpuscle level of 400 microgr % at birth rises to 1240 microgr % in adult life (*Acta Paediat* 41/Suppl 87/105pp/52). MAB Brazier & HK Beecher propose number of movements as index of depth of sleep in testing sedatives (*J Appl Physiol* 4/819/52). L Buchel & Co report pharmacological studies on phosphorus derivatives of adenosine (*J Physiologie* 44/173/52). GP Child & M Crump suggest toxicity of disulphiram due to irritant action & to inhibition of enzyme systems (*Acta Pharmacol Toxicol* 8/305/52). Note Colloquium on *Mikroskopische & Chemische Organisation der Zelle* (Springer, Berlin, 52, 106pp, M9.6). JJ Cordonnier finds testicular atrophy follows long giving of testosterone (*J Gerontol* 7/375/52). AF Essellier & KF Wagner show eosinopenia following ACTH depends on functioning of reticulo-endothelial system (*Acta Haematol* 8/63/52). HH Fox discusses chemical control of TB (*Science* 116/229/Aug 8/52). E Jacobsen analyses metabolism of ethanol

(*Pharmacol Rev* 4/107/52). K Kuriaki & H Shibue report on organ distribution & excretion of methanol (*Jap J Pharmacol* 1/114/52). O Mayrhofer studies succinyl cholinchloride, a new short acting muscle relaxant (*Anaesth* 1/9/52). P Nordquist shows hyaluronidase aids procaine penetration of nerve & demonstrates a procaine esterase (*Acta Pharmacol Toxicol* 8/195, 207, 217, 226, 233/52). JF Reinhard & Co describe pharmacology of isonicotinyl hydrazide, new antituberculinic (*Science* 116/166/Aug 15/52). C Remington reviews haems & porphyrins in health & disease (*Acta Med Scand* 143/161, 177/52). H Schaeffer offers thory of neuromuscular regulation of muscle tone (*Der Anaesth* 1/1/52). M. Suda & S Naona find ATP energy metabolism involved in enzyme transformation (*Med J Osaka Univ* 3/95/52). JEP Toman discusses neuropharmacology of peripheral nerves (*Pharmacol* Rev 4/168/52).

THE REST: B Davidson obits antivivisectionists (*Collier's* Aug 16/52). WC Davison edits history of Duke Univ health services (Duke Univ, Durham, NC, 52, 314pp). LI Dublin & HH Marks show increasing mortality among overweights (*Proc Asso Life Ins Med Dir Amer* 52/31pp). JWM DuMond & ER Cohen discuss fundamental atomic constants (*Amer Sci* 40/447/52). W Haynes edits (*Chemical Trade Names & Commercial Synonyms* (Macmillan, NY, 52, 320pp, $6). W Heubner deserves & receives Festschrift on 75th birthday (*Arch Exp Path Pharmakol* 215/1–216/52). LE Hinkle Jr & N Plummer explore life stress & industrial absenteeism (*Indust Med Surg* 21/363/52). J Jackson well edits *The Western Gate*, glamorizing San Francisco (Farrar Straus, NY, 52, 524pp, $4.75). LC McCabe edits *Air Pollution* (McGraw-Hill, NY, 52, 847pp, $12.5). JR Paul & colleagues well eulogize Francis Gilman Blake (1887–1952) (*Yale J Biol Med* 24/435–577/52). W Seagle apologizes for *Law: the Science of Inefficiency* (Macmillan, NY, 52, 235pp, $3.5). RD Tuddenham starts study of reputation by school children's evaluation of each other in setting up criteria for diagnosis of social adjustment (*Psych Monog* 333, 52, 58pp).

OCTOBER, 1952

GENERAL: C Baker scrutinizes *Hemingway: The Writer as an Artist*, & gives checklist of writings (Princeton Univ Press, 52, 340pp, $4.53. FR Barta discusses *The Moral Theory of Behavior* (CC Thomas Springfield, Ill, 52, 32pp, $2). Neat is LM Bayer & ES Green's *Kitchen Strategy: The Family Angle on Nutrition* (*ibid.* 112pp, $2.5). WR Brain fittingly discusses *Mind, Perception & Science* (*ibid.* 95pp, $2.5). CL Camp sings *Earth Song: A Prologue to History* (Univ California Press, Berkeley, 52, 168pp, $5). H Chapin & FGW Smith describe *The Ocean River* (Scribner's, NY, 52, 325pp, $3.5). E. Ferber's *Giant* amuses Texans (Doubleday, NY, 52, 448pp, $4). W Haymaker pictures *The Founders of Neurology* (CC Thomas, Springfield, Ill, 52, 460pp, $9.5). CA Lee offers *Instrumental Detection of Deception: The Lie Test* (*ibid*, 268pp, $6.5). M Levy develops social analysis in *The Structure of Society* (Princeton Univ Press,

52, 544pp, $6). Important ethically is A Montagu's *Darwin: Competition & Co-operation* (H Schuman, NY, 52, 148pp, $2.5). WS McCulloch is deterministic in *Finality & Form in Nervous Activity* (CC Thomas, Springfield, Ill, 52, 66pp, $3). JMD & EH Olmsted prepare fine account of *Claude Bernard & the Experimental Method in Medicine* (H Schuman, NY, 52, 277pp, $4). HB Shumacker Jr wisely reflects on medical education (*Surg* 32/161/52). Note Symposium on WS Halsted Centenary (*ibid*, pp 443–550). F Victor graphologizes in *Handwriting: A Personality Projection* (CC Thomas, Springfield, Ill, 52, 168pp, $3.75). H Weyl analyses *Symmetry* (Princeton Univ Press, 52, 176pp, $3.75). CEA Winslow reflects on *Man & Epidemics* (*ibid*, 256pp, $4). Congratulations to *Yale Journal of Biology & Medicine* on its 25th birthday, on which EH Thomson comments (25/2/52).

PRECLINICAL: G Asboe-Hansen finds cortisone decreases hyaluronic acid producing mast cells in connective tissue (*Proc Soc Exp Biol Med* 80/677/52). JT Bonner discusses *Morphogenesis* (Princeton Univ Press, 52, 304pp, $5). E Braun-Menendez notes desoxycorticosterone increases specific appetite for NaCl, protentiated by cortisone & BAL & inhibited by sex hormones & phenergan (*Rev. Soc Argent Biol* 28/15, 23/52). AA Christman reviews purine & pyrimidine metabolism (*Physiol Rev* 32/303/52). EJ Conway surveys *Biochemistry of Gastric Acid Secretion* (CC Thomas, Springfield, Ill, 52, 183pp, $4.5). Our JC Finerty discusses parabiosis in physiological research (*Physiol Rev* 32/277/52). HE Gierke & Co reveal physics of vibrations in living tissues (*J Appl Physiol* 4/886/52). F Haurowitz discusses mechanism of immunological response (*Biol Rev* 27/247/52). R Kenk & ML Nall offer annotated bibliography of physiology of brain circulation (*Physiol Rev* Suppl 1, July 52, 437pp, $4.5). CE Rapela & BA Houssay show that asphyxia increases adrenal secretion of epi & norepinephrim (*Rev Soc Argent Biol* 28/7/52). O Schaumann well reviews new synthetic analgesics (*Arch Exp Path Pharmakol* 216/48/52). E. Simonson & J Brozek discuss flicker frequency (*Physiol Rev* 32/359/52). Note Symposia on world food problems, secretion of electrolytes, alterations in pulse form, geriatric nutrition & antigenic variation of influenza viruses (*Fed Proc* 11/667, 695, 732, 775, 799/52). KM Smith reviews latency in viruses & origin of new virus diseases (*Biol Rev* 27/347/52). HK Zimmerman Jr surveys experimental estimation of solubilities (*Chem Rev* 51/25/52).

CLINICAL: GW Beebe & ME DeBakey offer *Battle Casualties: Incidence, Mortality & Logistic Considerations* (CC Thomas, Springfield, Ill, 52, 277pp, $7.5). VV Brunst suggests stimulation of growth by x-rays caused by disintegration products of hurt cells (*Am J Roentg Rad Therapy Nucl Med* 68/281/52). CF Code & Co analyse motility of alimentary canal (*Am J Med* 13/328/52). CC Congdon & Co find bone marrow stimulates hematopoiesis & aids recovery in radiation injury (*J Nat Cancer Inst* 13/73/52). RR Guehring & Co find reduced pantothenic acid intake reduces liver & serum cholesterol (*J Biol Chem* 197/485/52). Our CS Livingood

& JF Mullins describe management of bacterial skin infections (*Postgrad Med* 12/15/52). HW Newman & Co find maximum daily metabolism of alcohol is 3 to 4 gms/kg (*Science* 116/328/Sept 26/52). A Pekkarinen & L Aro recommend norepinephrin for shock (*Ann Clin Gyn Fenn* 41/69/52). F Schaub discusses macrogloublinanemia (*Schweiz Med Wchnschr* 82, 890/Sept 6/52). RF Shropshire & Co treat cataract with fish lens protein extract, following Tobias (*Science* 116/276/Sept 12/52). J Wedel & Co revascularize heart by pedicle skin flap (*ibid* p319, Sept 2652).

NOVEMBER, 1952

SUCCINYL-CHOLINE: First described by R Hunt & R Taveau (*Brit Med J* 2/1788/06), noted as depolarizing neuromuscular inhibitor by WDM Paton & EJ Zaimis (*Lancet* 2/568/50), its pharmacology is now analysed by S Thesleff (*Acta Physiol Scand* 26/104/52), & it is recommended as safe efficient muscular relaxant in shock therapy by D Bovet & F Bovet-Nitti (*Schw Med Wchnschr* 82/1009/Oct 4/52), & in anesthesia by FT Foldes & Co (*New Eng J Med* 247/596/Oct 16/52); a neat example of wide international medical cooperation.

GENERAL: RN Anshen edits *Moral Principles of Action* (Harper, NY, 52, 720pp, $7.5). LV Bertalanffy evaluates modern biological thought in *Problems of Life* (Wiley, NY, 52, 225pp, $4). WB Castle appraises contributions of George R. Minot (1885–1950) (*New Eng J Med* 247/585/Oct 16/52). EC Cherry considers the communication of information (*Am Sci* 40/640/52). HT Clarke eulogizes Henry D. Dakin (1880–1952) (*J Biol Chem* 198/491/52). P Cloake discusses brain function & "mind" (*Brain* 75/273/52). A Grunbaum discusses causality & human behavior (*Am Sci* 40/665/52). A Macbeath offers Gifford Lectures on nature of ethics in *Experiments in Living* (Macmillan, London, 52, 352pp, 30s). CF Mayer reviews history of multiparous births (*Acta Gen Med Gem* 1/118/242/52). PB Medawar biologically analyses individuality (*Am Sci* 40/632/52). WT Stace surveys *Religion & the Modern Mind* (Lippincott, Phila, 52, 285pp, $3.75). ID Suttie reveals *The Origins of Love & Hate* (Julian, NY, 52, 225pp, $4). S Rapport & H Wright revive *Great Adventures in Medicine* (Dial, NY, 52, 895pp, $5). M Waterstreet & BR James collect *Adlai's Almanac: The Wit & Wisdom of Stevenson of Illinois* (Schuman, NY, 52, 220pp, $4).

CLINICAL: WB Daniels & FG MacMurray review non-bacterial cat scratch disease (*Ann Int Med* 37/697/52). D Denny-Brown discusses nature of muscular disease (*Canad Med Asso J* 67/1/52). R Domenjos indicates inhibitory effect of isonicotinyl hydrazide on inflammatory reactions (*Schw Med Wchnschr* 82/1023/Oct 4/52). P Fatt & B Katz find sodium deficiency disposes to neuromuscular block (*J Physiol* 118/73/52). Our L Gregory & Co confirm value of octamethyl-pyrophosphate in myasthenia gravis (*Am J Med* 13/423/52). JD Hardy, HG Wolff & H Goodell offer *Pain Sensations & Reactions* (Wms & Wilkins, Balt, 52, 450pp, $9.5). CA Hernberg notes cortisone inhibition of chemical irritation (*Ann Med Int Fenn*

41/50/52). S Hotta & IL Chaikoff think early vascular degeneration in diabetes due to increased rate of liver cholesterol formation (*J Biol Chem* 198/895/52). J Landau & Co report decrease in dark adaptation in 4th to 7th month of human pregnancy (*Acta Med Orient* 11/121/52). RB McConnell & Co say excessive cigarette smoking may help lung cancer (*Lancet* 2/651/Oct 4/52). L Meyler reports *Side Effects of Drugs* (Elsevir, Houston, 52, 280pp, $3.5). E Mundt describes reticuloendotheliosis with poor prognosis & therapy (*Ztschr klin Med* 149/599/52). D Muting continues studies on methionin imbalance & therapy in liver disease (*ibid* p 566). O Storstein emphasizes value of direct oxygen administration in anoxemia (*Acta Med Scand*, Suppl 269, Bergen, 52, 185pp). MK Polano reviews *Skin Therapeutics* (Elzevier, Houston, 52, 276pp, $6.5). FH Smirk says hypotensive action of subcut injection of hexamethonium cmpds may be prolonged several hours by dissolving in 25% polyvinyl-pyrrolidone (*Lancet* 2/695/Oct 11/52). BF Smith offers *Manual of Electrocardiography* (Elzevier, Houston, 52, 215pp, $4.5). WHV Wyss reviews endocarditis, emphasizing exclusive bacterial etiology (*Helv Med Acta* 19/17/52).

Basic: WE Balfour & C Hebb show co-enzyme A necessary for acetylcholine synthesis either from acetate or citrate (*J Physiol* 118/94/52). HF Cook analyses pain threshold for microwave & infrared radiation (*ibid* p 1). A Dohrn & H Rein note a spleen effect on heart (*Arch ges Physiol* 255/448/52). E Fluckiger & F Verzar note body temperature fall at low atmospheric pressure, with adaptive delayed increased heat production mediated by hypothlamus & adrenal cortex (*Helv Physiol Pharmacol Acta* 10/349/52). M Goffart find adrenalin & splanchnic stimulation increase contractile tension of curarized mammalian muscle independent of blood pressure or potassium liberation (*Arch Internat Physiol* 60/318–418/52). EJ Harris & M Maizies describe distribution of ions in suspensions of red blood cells (*J Physiol* 118/40/52). E Helmreich & S Goldschmidt describe "corhormon," a mg containing heart extract as coferment activating adenosine triphosphate dephosphorilizing system (*Ztschr Physiol Chem* 290/119/52). G Jongebreuer reports on biochemorphology of pyrones & khellin (*Arch Internat Pharmaco* (90/384/52). M Keeri-Szanto discusses fluidity of circulation in re intravascular clotting (*Rev Belge Path Med Exp* 22/103/52). M Terziolglu & Co note adjustments in blood physiology to mid altitudes (*Arch Internat Physiol* 60/233, 253/52).

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